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13. ABSTRACT *(Maximum 200 Words)*

In breast tumor cells (e.g. SP1 cells), the guanine nucleotide exchange factor (GEF, the dbl or DH family), Tiam1 (T lymphoma invasion and metastasis) is detected as a 200 kDa protein. Tiam1 is capable of catalyzing GDP/GTP exchange for Rac1. In particular, the aa393-aa738 sequence of Tiam1, which contains the NH2-terminal pleckstrin homology (PHn), a putative coiled coil region (CC) and an additional adjacent region (Ex) (designated as PHn-CC-Ex domain of Tiam1), is involved in the direct interaction with CD44v3 isoform (the hyaluronan receptor) and ankyrin (the cytoskeletal protein) both *in vivo* and *in vitro*. Specifically, the binding of HA to the CD44v3 isoform stimulates Tiam1-specific GDP/GTP exchange for Rho-like GTPases such as Rac1, and promotes cytoskeleton-mediated breast tumor cell migration. Furthermore, we have demonstrated that the PHn-CC-Ex domain of Tiam1 contains an ankyrin binding site. The structural homology between the ankyrin binding domain of Tiam1 (the sequence between aa717 and aa727 within the PHn-CC-Ex domain) and CD44 is quite striking. Most importantly, the Tiam1-ankyrin interaction also promotes Rac1 activation and breast tumor cell migration. These observations clearly indicate that Tiam1 contains multiple functional domains (e.g. a CD44-specific membrane localization site and a cytoskeleton binding region for ankyrin) required for the regulation of Tiam1-Rac1 signaling and cytoskeleton function during metastatic breast tumor progression.

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FOREWORD

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Billy Y.W. Bourgeois 7/31/01

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INTRODUCTION

The activity of Rho-like GTPases is modulated by specific guanine-nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), that provide a specific activating state for Rho-like GTPases (1). In particular, several guanine nucleotide exchange factors (GEFs - the dbl or DH family) have been identified as oncogenes due to their ability to up-regulate RhoGTPase activity leading to malignant transformation (1). One of these GEFs is an invasion and metastasis-specific protein, Tiam1 (T lymphoma invasion and metastasis) which was identified by retroviral insertional mutagenesis and selection for invasive cells in vitro (2). Overexpression of both N- and C-terminally truncated, as well as full-length Tiam1 proteins, induced the invasive phenotype in otherwise noninvasive cells. Moreover, such invasive behavior correlated with metastasis formation in animal models (2). Structurally, Tiam1 is an approximately 200 kD protein which is largely hydrophilic and, by sequence homology, contains numerous functional domains found in signal transduction proteins (2,3). A C-terminal region of 240 amino acids contains significant sequence homology to the Dbl-homologous domain (DH) of many proteins which exhibit GDP-GTP exchange activity for specific members of the Ras superfamily of GTP-binding proteins (4,5). In vitro, Tiam1 has indeed been shown to act as an exchange protein for the Rho subfamily of GTPases, including Rac1, Cdc42, and RhoA (6). In addition, Tiam1 contains two pleckstrin homology domains (PH) which are commonly detected in signaling molecules and cytoskeletal proteins (7). It may also mediate association with the submembrane region of the cell via protein-protein or protein-lipid interactions (7). Between the N-terminal PH domain and the DH domain is a region with sequence homology to the Discs-large homologous region (DHR) of the drosophila tumor suppressor protein (8). Although its function is unknown, this region may facilitate interaction with the submembrane cytoskeleton (9). Finally, the Tiam1 amino terminus contains a potential myristoylation signal (2) which may be involved in membrane association. Taken together, sequence analysis of Tiam1 suggests that its association with the invasive/metastatic phenotype may be mediated via submembrane cytoskeletal regulation and/or activation of Rho family GTPases. Tiam1 expression has been detected in numerous cell lines, including breast tumor cells (3). However, the question whether Tiam1-catalyzed Rho GTPase activation contributes to invasive properties of breast tumor cells is addressed in this study.

BODY

(A) CD44 INTERACTION WITH TIAM1 PROMOTES Rac1 SIGNALING AND CYTOSKELETON-MEDIATED BREAST TUMOR CELL INVASION AND MIGRATION

In this study we have explored the interaction between CD44 [the hyaluronan (HA) receptor] and Tiam1 (a guanine nucleotide exchange factor) in metastatic breast tumor cells (SP-1 cell line). Immunoblot and immunoprecipitation analyses indicate that both the CD44v3 isoform (e.g.$CD44v_{3,8\text{-}10}$) and the Tiam1 protein are expressed in SP-1 cells. and that these two proteins are physically associated as a complex in vivo. Using E. coli-derived calmodulin-binding peptide (CBP)-tagged the NH2-terminal pleckstrin homology (PHn) domain and an adjacent protein interaction domain (designated as PHn-CC-Ex, aa393-aa738) of Tiam1 and an in vitro binding assay, we have detected a specific binding interaction between the Tiam1's PHn-CC-Ex and CD44.

Scatchard plot analysis indicates that there is a single high affinity CD44 binding site in Tiam1's PHn-CC-Ex with an apparent dissociation constant (Kd) of 1nM which is comparable to CD44 binding (Kd ≈2nM) to intact Tiam1. Biochemical studies, using competition binding assays and a synthetic peptide identical to "GELPNPKRLL" [a sequence between aa630 and aa640 of Tiam1's PHn-CC-Ex], demonstrate that this 11 amino acid peptide (but not a scrambled peptide) binds specifically to the cytoplasmic domain of CD44. This peptide also competes effectively for Tiam1 binding to CD44. These findings suggest that the PHn-CC-Ex domain is the primary Tiam1 binding region for CD44. Most importantly, the binding of HA to CD44v3 of SP-1 cells stimulates Tiam1-catalyzed Rac1 signaling and cytoskeleton-mediated tumor cell invasion and migration.

Further analyses show transfection of SP-1 cells with Tiam1cDNA results in an upregulation of Rac1 signaling and cytoskeleton-mediated breast tumor cell invasion/ migration in a HA and CD44v3-dependent manner. Finally, we have constructed a Tiam1 deletion mutant cDNA (lacking the CD44 binding site) and expressed it in SP-1 cells. Our results indicate that this Tiam1 deletion mutant effectively inhibits HA/CD44v3-mediated tumor cell behaviors. These findings strongly suggest that CD44v3 isoform plays an important role in linking Tiam1 to the plasma membrane which is required for HA stimulated oncogenic signaling and cytoskeleton-mediated tumor cell invasion/migration during breast cancer progression.

(B) ANKYRIN-TIAM1 INTERACTION PROMOTES RAC1 SIGNALING AND METASTATIC BREAST TUMOR CELL INVASION AND MIGRATION

Tiam1 (T lymphoma invasion and metastasis) is one of the known guanine nucleotide (GDP/GTP) exchange factors (GEFs) for RhoGTPases (e.g. Rac1) and is expressed in breast tumor cells (e.g. SP-1 cell line). Immunoprecipitation and immunoblot analyses indicate that Tiam1 and the cytoskeletal protein, ankyrin, are physically associated as a complex in vivo. In particular, the ankyrin repeat domain (ARD) of ankyrin is responsible for Tiam1 binding. Biochemical analyses indicate that the 11aa sequence between aa717 and aa727 of Tiam1 (^{717}GEGTDAVKRS^{727}L) is the ankyrin binding domain. Most importantly, ankyrin binding to Tiam1 activates GDP/GTP exchange on RhoGTPases (e.g. Rac1).

Using an Escherichia coli-derived calmodulin-binding peptide (CBP)-tagged recombinant Tiam1 (aa393-aa728) fragment which contains the ankyrin-binding domain, we have detected a specific binding interaction between the Tiam1 (aa393-aa738) fragment and ankyrin in vitro. This Tiam1 fragment also acts as a potent competitive inhibitor for Tiam1 binding to ankyrin. Transfection of SP-1 cell with Tiam1 cDNAs stimulates (i)Tiam1-ankyrin association in the membrane projections, (ii) Rac1 activation and (iii) breast tumor cell invasion and migration. Co-transfection of SP1 cells with green fluorescent protein (GFP)-tagged Tiam1 fragment cDNA and Tiam1 cDNA effectively blocks Tiam1-ankyrin co-localization in the cell membrane and inhibits GDP/GTP exchange on Rac1 by ankyrin-associated Tiam1 and tumor-specific phenotypes. These findings suggest that ankyrin-Tiam1 interaction plays a pivotal role in regulating Rac1 signaling and cytoskeleton function required for oncogenic signaling and metastatic breast tumor cell progression.

KEY RESEARCH ACCOMPLISHMENTS:

•We have found that Tiam1 (T lymphoma invasion and metastasis) is one of the known guanine nucleotide (GDP/GTP) exchange factors (GEFs) for RhoGTPases (e.g. Rac1) and is expressed in breast tumor cells (e.g. SP-1 cell line).

(A) Tiam1 Interaction With The Metastasis-Specific Molecule, CD44 [the hyaluronan (HA) receptor]:

•We have explored the interaction between the metastasis-specific molecule, CD44 [the hyaluronan (HA) receptor] and Tiam1 (a guanine nucleotide exchange factor) in metastatic breast tumor cells (SP1 cell line).

•Scatchard plot analysis indicates that there is a single high affinity CD44 binding site in Tiam1's PHn-CC-Ex domain with an apparent dissociation constant (Kd) of 0.2nM which is comparable to CD44 binding (Kd ≈0.13nM) to intact Tiam1. These findings suggest that the PHn-CC-Ex domain is the primary Tiam1 binding region for CD44.

•Most importantly, the binding of HA to CD44v3 of SP1 cells stimulates Tiam1-catalyzed Rac1 signaling and cytoskeleton-mediated tumor cell migration.

•Transfection of SP1 cells with Tiam1cDNA promotes Tiam1 association with CD44v3 and upregulates Rac1 signaling as well as HA/CD44v3-mediated breast tumor cell migration. Co-transfection of SP1 cells with PHn-CC-Ex cDNA and Tiam1 cDNA effectively inhibits Tiam1 association with CD44 and efficiently blocks tumor behaviors.

•These observations clearly suggest that Tiam1 contains multiple functional domains [e.g. membrane localization site(s) and cytoskeleton binding domains] required for the regulation of Tiam1-Rac1 signaling and cytoskeleton function leading to metastatic breast tumor cell progression.

(B)Tiam1 Interaction With the Cytoskeletal Protein, Ankyrin:

•Tiam1 and the cytoskeletal protein, ankyrin, are physically associated as a complex in vivo.

•The ankyrin repeat domain (ARD) of ankyrin is responsible for Tiam1 binding; the 11aa sequence between aa717 and aa727 of Tiam1 (^{717}GEGTDAVKRS^{727}L) is the ankyrin binding domain.

•Most importantly, ankyrin binding to Tiam1 activates GDP/GTP exchange on RhoGTPases (e.g. Rac1).

•Using an Escherichia coli-derived calmodulin-binding peptide (CBP)-tagged recombinant Tiam1 (aa393-aa728) fragment which contains the ankyrin-binding domain, we have detected a specific binding interaction between the Tiam1 (aa393-aa738) fragment and ankyrin in vitro. This Tiam1

fragment also acts as a potent competitive inhibitor for Tiam1 binding to ankyrin.

•Transfection of SP-1 cell with Tiam1 cDNAs stimulates (i)Tiam1-ankyrin association in the membrane projections, (ii) Rac1 activation and (iii) breast tumor cell invasion and migration. Co-transfection of SP1 cells with green fluorescent protein (GFP)-tagged Tiam1 fragment cDNA and Tiam1 cDNA effectively blocks Tiam1-ankyrin co-localization in the cell membrane and inhibits GDP/GTP exchange on Rac1 by ankyrin-associated Tiam1 and tumor-specific phenotypes.

REPORTABLE OUTCOMES:

•Manuscripts and abstracts: see section #13 (final report section).

•Funding applied for based on work supported by this award:

Funded Active Grants:

NCI Grant (2000-2005)"CD44/Variant-Cytoskeleton In Breast Cancer Progression".

NCI Grant (1999-2003) "CD44-p185^{HER2} Interaction In Ovarian Cancer Progression".

NCI Grant (2001-2003) "CD44 Interaction With Cytokine Receptors in Breast Cancer Bone Metastasis".

US Army Breast Cancer Grant (DOD) (1999-2002) "A Novel Signaling Perturbation and Ribozyme Gene Therapy Proceudre to Block Rho-Kinase (ROK) Activation and Breast Tumor Metastasis".

CONCLUSIONS:

Signaling to the RacGTPase is known to regulate actin assembly associated with membrane ruffling, pseudopod extension, cell motility and cell transformation (10,11) and has been shown to be abnormal in transformed cells (6). The fact that Rac1 induces stress fiber formation in a Rho-dependent manner indicates that "cross-talk" occurs between the Rho and Rac1 signaling pathways (11).

Several guanine nucleotide exchange factors (GEFs, the dbl or DH family) have been identified as oncogenes by their ability to up regulate RhoGTPase activity during malignant transformation (6,10). One of these GEFs is Tiam1 (T lymphoma invasion and metastasis), which was identified by retroviral insertional mutagenesis and selected for its invasive cell behavior *in vitro* (2). It has been clearly shown to regulate Rac1 activation (2,3). Tiam1 is a largely hydrophilic molecule that contains several functional domains, including a Dbl homology (DH) domain, a Discs-large homology region (DHR), and two pleckstrin homology (PH) domains (PHn, the PH domain located at the NH2-terminal region of the molecule; and PHc, the PH domain located at the COOH-terminal

region of the molecule) (2). The DH domain of Tiam1 exhibits GDP/GTP exchange activity for specific members of the Ras superfamily of GTP-binding proteins (2) and plays an important role in Rac1 signaling and cellular transformation (2,3). In breast tumor cells (e.g. SP1 cells), Tiam1 is detected as a 200 kDa protein that is capable of catalyzing GDP/GTP exchange for Rac1 as described for Tiam1 in other cell types (12,13). Recently, we have presented new evidence that a close interaction occurs both *in vivo* and *in vitro* between Tiam1 and the CD44v3 isoform. The $CD44v_3$ isoform is closely associated with Tiam1 aa393-aa738 containing the NH2-terminal pleckstrin homology (PHn), a putative coiled coil region (CC) and an additional adjacent region (Ex) (designated as PHn-CC-Ex domain of Tiam1) in breast tumor cells (12). Most importantly, HA binding to the $CD44v_3$ isoform stimulates Tiam1-specific GDP/GTP exchange for Rho-like GTPases such as Rac1, and promotes cytoskeleton-mediated tumor cell migration (12). These findings are consistent with a study by Oliferenko, et al (14) showing that Rac1 activation can be induced by HA binding to CD44.

A recent study in our laboratory has demonstrated that the PHn-CC-Ex domain of Tiam1 also contains an ankyrin binding site (13). The structural homology between the ankyrin binding domain of Tiam1 (the sequence between aa717 and aa727 within the PHn-CC-Ex domain) and CD44 is quite striking. Most importantly, the Tiam1-ankyrin interaction promotes Rac1 activation and breast tumor cell migration (13). These observations clearly suggest that Tiam1 contains multiple functional domains (e.g. a CD44-specific membrane localization site and a cytoskeleton binding region for ankyrin) required for the regulation of Tiam1-Rac1 signaling and cytoskeleton function. Taken together, these results suggest that Tiam1 interaction with CD44v3 and ankyrin plays a pivotal role in regulating Rac1-activated oncogenic signaling and cytoskeleton-mediated metastatic breast tumor progression.

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APPENDICES: (see attached reprints and previous scientific evaluations).

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CD44 Interaction with Tiam1 Promotes Rac1 Signaling and Hyaluronic Acid-mediated Breast Tumor Cell Migration*

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In this study we have explored the interaction between CD44 (the hyaluronic acid (HA)-binding receptor) and Tiam1 (a guanine nucleotide exchange factor) in metastatic breast tumor cells (SP1 cell line). Immunoprecipitation and immunoblot analyses indicate that both the CD44v3 isoform and the Tiam1 protein are expressed in SP1 cells and that these two proteins are physically associated as a complex *in vivo*. Using an *Escherichia coli*-derived calmodulin-binding peptide-tagged Tiam1 fragment (*i.e.* the NH_2-terminal pleckstrin homology (PHn) domain and an adjacent protein interaction domain designated as PHn-CC-Ex, amino acids 393–738 of Tiam1) and an *in vitro* binding assay, we have detected a specific binding interaction between the Tiam1 PHn-CC-Ex domain and CD44. Scatchard plot analysis indicates that there is a single high affinity CD44 binding site in the PHn-CC-Ex domain of Tiam1 with an apparent dissociation constant (K_d) of 0.2 nM, which is comparable with CD44 binding (K_d = ~0.13 nM) to intact Tiam1. These findings suggest that the PHn-CC-Ex domain is the primary Tiam1-binding region for CD44. Most importantly, the binding of HA to CD44v3 of SP1 cells stimulates Tiam1-catalyzed Rac1 signaling and cytoskeleton-mediated tumor cell migration. Transfection of SP1 cells with Tiam1cDNA promotes Tiam1 association with CD44v3 and up-regulates Rac1 signaling as well as HA/CD44v3-mediated breast tumor cell migration. Co-transfection of SP1 cells with PHn-CC-Ex cDNA and Tiam1 cDNA effectively inhibits Tiam1 association with CD44 and efficiently blocks tumor behaviors. Taken together, we believe that the linkage between CD44v3 isoform and the PHn-CC-EX domain of Tiam1 is required for HA stimulated Rac1 signaling and cytoskeleton-mediated tumor cell migration during breast cancer progression.

The transmembrane glycoprotein CD44 isoforms are all major hyaluronic acid (HA)[1] cell surface receptors that exist on many cell types, including macrophages, lymphocytes, fibroblasts, and epithelial cells (1–6). Because of their widespread occurrence and their role in signal transduction, CD44 isoforms have been implicated in the regulation of cell growth and activation as well as cell-cell and cell-extracellular matrix interactions (1–7). One of the distinct features of CD44 isoforms is the enormous heterogeneity in the molecular masses of these proteins. It is now known that all CD44 isoforms are encoded by a single gene that contains 19 exons (8). Of the 19 exons, 12 exons can be alternatively spliced (8). Most often, the alternative splicing occurs between exons 5 and 15, leading to an insertion in tandem of one or more variant exons (v1–v10 (exon 6-exon 14) in human cells) within the membrane-proximal region of the extracellular domain (8). The variable primary amino acid sequence of different CD44 isoforms is further modified by extensive *N*- and *O*-glycosylations and glycosaminoglycan additions (9–12). In particular, CD44v3-containing isoforms have a heparin sulfate addition at the membrane-proximal extracellular domain of the molecule that confers the ability to bind heparin sulfate-binding growth factors (9, 10). Cell surface expression of CD44v isoforms changes profoundly during tumor metastasis, particularly during the progression of various carcinomas including breast carcinomas (13–17). In fact, CD44v isoform expression has been used as an indicator of metastasis.

It has been shown that interaction between the cytoskeletal protein, ankyrin, and the cytoplasmic domain of CD44 isoforms plays an important role in CD44 isoform-mediated oncogenic signaling (6, 18, 19). Specifically, the ankyrin-binding domain (*e.g.* NGGNGTVEDRKPSEL between amino acids 306 and 320 in the mouse CD44 (20) and NSGNGAVEDRKPSGL amino acids 304 and 318 in human CD44 (21)) is required for the recruitment of Src kinase and the onset of tumor cell transformation (21). Furthermore, HA binding to CD44 stimulates a concomitant activation of $p185^{HER2}$-linked tyrosine kinase (linked to CD44s via a disulfide linkage) and results in a direct cross-talk between two different signaling pathways (*e.g.* proliferation *versus* motility/invasion) (22). In tumor cells, the transmembrane linkage between CD44 isoform and the cytoskeleton promotes invasive and metastatic-specific tumor phenotypes (*e.g.* matrix degradation (matrix metalloproteinases) activities (23, 24), "invadopodia" formation (membrane projections), tumor cell invasion, and migration) (23). These findings strongly suggest that the interaction between CD44 isoform and the cytoskeleton plays a pivotal role in the onset of oncogenesis and tumor progression.

The Rho family proteins (*e.g.* Rho, Rac, and Cdc42) are members of the Ras superfamily of GTP-binding proteins structurally related to but functionally distinct from Ras itself (25, 26). They are associated with changes in the membrane-linked cytoskeleton (26). For example, activation of RhoA, Rac1, and Cdc42 have been shown to produce specific structural changes in the plasma membrane-cytoskeleton reorganization leading

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[1] The abbreviations used are: HA, hyaluronic acid; PHn, pleckstrin homology; PHc, PH domain located at the COOH-terminal region of the molecule; CC, coiled coil region; Ex, extra region; CBP, calmodulin-binding peptide; GFP, green fluorescent protein; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; Rh, rhodamine; FITC, fluorescein isothiocyanate.

to membrane ruffling, lamellipodia, filopodia, and stress fiber formation (26). The coordinated activation of these GTPases is considered to be a possible mechanism underlying cell motility, an obvious prerequisite for metastasis (27–29). In particular, Rac1 activation is known to initiate oncogenic signaling pathways that promote cell shape changes (33, 34), influence actin cytoskeleton organization (33, 34), and stimulate gene expression (35–37). The question of whether Rac1 activation is also involved in CD44v3-related cytoskeleton function that results in the metastatic phenotypes (*e.g.* tumor cell migration) of breast tumor cells remains to be answered.

Tiam1 (T lymphoma invasion and metastasis 1) has been identified as an oncogene because of its ability to activate Rho-like GTPases during malignant transformation (38, 39). Specifically, Tiam1 is capable of activating Rac1 *in vitro* as a guanine nucleotide exchange factor and inducing membrane cytoskeleton-mediated cell shape changes, cell adhesion, and cell motility (34, 40–42). It also acts as a Rac-specific guanine nucleotide exchange factor *in vivo* and induces an invasive phenotypes in lymphoma cells (40). These findings have prompted several research groups to investigate the mechanisms involved in the regulation of Tiam1. For example, addition of certain serum-derived lipids (*e.g.* sphingosine-1-phosphate and lysophosphatidic acid) to T-lymphoma cells promotes Tiam1-mediated Rac1 and Cdc42 signaling and T-lymphoma cell invasion (43). Tiam1 has also been found to be phosphorylated by protein kinase C in Swiss 3T3 fibroblasts stimulated by lysophosphatidic acid (44) and platelet-derived growth factor (45). Most recently, Exton and co-workers (46) demonstrate that phosphorylation of Tiam1 by Ca^{2+}/calmodulin-dependent protein kinase II (but not protein kinase C) regulates Tiam1-catalyzed GDP/GTP exchange activity *in vitro*. These findings support the notion that posttranslational modifications of Tiam1 by certain serine/threonine kinase(s) during surface receptor-mediated activation may play an important role in Tiam1-Rac1 signaling. Tiam1 transcript has been detected in breast cancer cells (39). However, it is not known at the present time whether there is any structural and functional relationship(s) between Tiam1-Rac1 signaling and CD44v3-mediated invasive and metastatic processes of breast cancer cells.

In this paper, using a variety of biochemical, molecular biological, and immunocytochemical techniques, we have found that the cell adhesion molecule, $CD44v_3$ isoform, which binds directly to HA, is closely associated with Tiam1 (in particular, the NH_2-terminal pleckstrin homology (PHn), a putative coiled coil region (CC), and an additional adjacent region (Ex), designated as PHn-CC-Ex domain of Tiam1) in SP1 breast tumor cells. Most importantly, HA binding to $CD44v_3$ isoform stimulates Tiam1-specific GDP/GTP exchange for Rho-like GTPases such as Rac1 and promotes cytoskeleton-mediated tumor cell migration. These findings suggest that a transmembrane interaction between CD44v3 and Tiam1 plays an important role in promoting oncogenic signaling and tumor cell-specific phenotypes required for HA-mediated breast tumor cell migration.

MATERIALS AND METHODS

Cell Culture—Mouse breast tumor cells (*e.g.* SP1 cell line) (provided by Dr. Bruce Elliott, Department of Pathology, and Biochemistry, Queen's University, Kingston, ON, Canada) were used in this study. Specifically, SP1 cell line was derived from a spontaneous intraductal mammary adenocarcinoma that arose in a retired female CBA/J breeder in the Queen's University animal colony. These cells were capable of inducing lung metastases by sequential passage of SP1 cells into mammary gland (47). These cells were cultured in RPMI 1640 medium supplemented with 5–7% fetal calf serum, folic acid (290 mg/liter), and sodium pyruvate (100 mg/liter). COS-7 cells were obtained from American Type Culture Collection and grown routinely in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% glutamine, 1% penicillin, and 1% streptomycin.

Antibodies and Reagents—For the preparation of polyclonal rabbit anti-Tiam1 antibody or rabbit anti-CD44v3 antibody, specific synthetic peptides (≈15–17 amino acids unique for the COOH-terminal sequence of Tiam1 or the CD44v3 sequence) were prepared by the Peptide Laboratories of Department of Biochemistry and Molecular Biology using an Advanced Chemtech automatic synthesizer (model ACT350). These Tiam1-related or CD44v3-related polypeptides were conjugated to polylysine and subsequently injected into rabbits to raise the antibodies, respectively. The anti-Tiam1-specific or anti-CD44v3-specific antibody was collected from each bleed and stored at 4 °C containing 0.1% azide. The anti-Tiam1 IgG or anti-CD44v3 IgG fraction was prepared by conventional DEAE-cellulose chromatography, respectively. Mouse monoclonal anti-HA (hemagglutinin epitope) antibody (clone 12 CA5) was purchased from Roche Molecular Biochemicals. Mouse monoclonal anti-green fluorescent protein (GFP) was purchased from PharMingen. *Escherichia coli*-derived GST-tagged Rac1 was kindly provided by Dr. Richard A. Cerione (Cornell University, Itheca, NY).

Cell Surface Labeling Procedures—SP1 cells suspended in PBS were surface labeled using the following biotinylation procedure. Briefly, cells (10^7 cells/ml) were incubated with sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce) (0.1 mg/ml) in labeling buffer (150 μM NaCl, 0.1 M HEPES, pH 8.0) for 30 min at room temperature. Cells were then washed with PBS to remove free biotin. Subsequently, the biotinylated cells were used for anti-CD44v3-mediated immunoprecipitation as described previously (23). These biotinylated materials precipitated by anti-CD44v3 antibody were analyzed by SDS-polyacrylamide gel electrophoresis, transferred to the nitrocellulose filters, and incubated with ExtrAvidin-peroxidase (Sigma). After an addition of peroxidase substrate (Pierce), the blots were developed using ECL chemiluminescence reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Immunoprecipitation and Immunoblotting Techniques—SP1 cells were solubilized in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 buffer and immunoprecipitated using rabbit anti-CD44v3 antibody or rabbit anti-Tiam1 antibody followed by goat anti-rabbit IgG, respectively. The immunoprecipitated material was solubilized in SDS sample buffer, electrophoresed, and blotted onto the nitrocellulose. After blocking nonspecific sites with 3% bovine serum albumin, the nitrocellulose filter was incubated with rabbit anti-Tiam1 antibody (5 μg/ml) or rabbit anti-CD44v3 antibody (5 μg/ml), respectively, for 1 h at room temperature followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution) at room temperature for 1 h. The blots were developed using ECL chemiluminescence reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

In some experiments, SP1 cells or COS cells (*e.g.* untransfected or transfected by various Tiam1 cDNAs including the full-length mouse Tiam1cDNA (FL1591) or HA-tagged NH_2-terminally truncated C1199 Tiam1cDNA or GFP-tagged PHn-CC-ExcDNA or C1199Taim1cDNA plus GFP-tagged PHn-CC-ExcDNA (as co-transfection) or vector only) were immunoblotted with mouse anti-HA antibody (5 μg/ml) or anti-GFP antibody (5 μg/ml), respectively, for 1 h at room temperature followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-mouse IgG (1:10,000 dilution) at room temperature for 1 h. The blots were developed using ECL chemiluminescence reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Cloning, Expression, and Purification of CD44 Cytoplasmic Domain (CD44cyt) from E. coli—The procedure for preparing the fusion protein of the cytoplasmic domain of CD44 was the same as described previously (48). Specifically, the cytoplasmic domain of human CD44 (CD44cyt) was cloned into pFLAG-AST using the PCR-based cloning strategy. Using human CD44 cDNA as template, one PCR primer pair (left, FLAG-*Eco*RI; right, FLAG-*Xba*I) was designed to amplify complete CD44 cytoplasmic domain. The amplified DNA fragments were one-step cloned into a pCR2.1 vector and sequenced. Then the DNA fragments were cut out by double digestion with *Eco*RI and *Xba*I and subcloned into *Eco*RI/*Xba*I double-digested pFLAG-AST (Eastman Kodak Co., Rochester, NY) to generate FLAG-pCD44cyt construct. The nucleotide sequence of FLAG/CD44cyt junction was confirmed by sequencing. The recombinant plasmids were transformed to BL21-DE3 to produce FLAG-CD44cyt fusion protein. The FLAG-CD44cyt fusion protein was further purified by anti-FLAG M2 affinity gel column (Eastman Kodak Co.). The nucleotide sequence of primers used in this cloning protocol are: FLAG-*Eco*RI, 5′-GAGAATTCGAACAGTCGAAGAAGGTGTCTCTTAAGC-3′, and FLAG-*Xba*I, 5′-AGCTCTAGATTACACCCCAATCTTCAT-3′.

Expression Constructs—Both the full-length mouse Tiam1cDNA

(FL1591) and the NH_2-terminally truncated Tiam1cDNA (C1199) were kindly provided by Dr. John G. Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Specifically, the full-length Taim1 (FL1591) cDNA was cloned into the eukaryotic expression vector, pMT2SM. The NH_2-terminally truncated C1199 Tiam1 cDNA (carrying a HA epitope tag at the 3′ end) was cloned into the eukaryotic expression vector, pUTSV1 (Eurogentec, Belgium). The Tiam1 fragment, PHn-CC-Ex domain was cloned into calmodulin-binding peptide (CBP)-tagged vector (pCAL-n) (Stratagen) using the PCR-based cloning strategy. Using human Tiam1 cDNA as a template, PHn-CC-Ex domain was amplified by PCR with two specific primers (left, 5′-AACTCGAGATGAGTACCACCAACAGTGAG-3′, and right, 5′-AAAAAGCTTTCAGCCATCTGGAACAGTGTCATC-3′) linked with specific enzyme digestion site (*Xho*I or *Hin*dIII). PCR product digested with *Xho*I and *Hin*dIII was purified with QIAquick PCR Purification Kit (Qiagen). The PHn-CC-Ex domain cDNA fragment was cloned into pCAL-n vector digested with *Xho*I and *Hin*dIII. The inserted PHn-CC-Ex domain sequence was confirmed by nucleotide sequencing analyses. The recombinant plasmids were transformed to BL21-DE3 to produce CBP-tagged PHn-CC-Ex fusion protein. This fusion protein was purified from bacteria lysate by calmodulin affinity resin column (Sigma).

The PHn-CC-Ex domain cDNA fragment was also cloned into pEGFPN1 vector (CLONTECH) digested with *Xho*I and *Hin*dIII to create GFP-tagged PHn-CC-Ex cDNA. The inserted PHn-CC-Ex domain sequence was confirmed by nucleotide sequencing analyses. This GFP-tagged PHn-CC-Ex domain cDNA was then used for transient expression in SP1 cells as described below. The molecular mass of the GFP-tagged PHn-CC-Ex is expressed as 68 kDa in SP1 or COS-7 cells by SDS-polyacrylamide gel electrophoresis and immunoblot analyses.

Cell Transfection—To establish a transient expression system, SP1 cells (or COS-7 cells) were transfected with various plasmid DNAs (*e.g.* HA-tagged C1199 Tiam1cDNA, GFP-tagged PHn-CC-ExcDNA, or HA-tagged C1199Tiam1cDNA plus GFP-tagged PHn-CC-ExcDNA (as co-transfection) or vector alone) using electroporation methods according to those procedures described previously (74). Briefly, SP1 cells were plated at a density of 2×10^6 cells/100-mm dish and transfected with 25 μg/dish plasmid cDNA using electroporation at 230 V and 960 microfaraday with a Gene Pulser (Bio-Rad). Transfected cells were grown in the culture medium for at least 24–48 h. Various transfectants were then analyzed for their protein expression (*e.g.* Tiam1-related proteins) by immunoblot, GDP/GTP exchange reaction on Rac1, and tumor cell migration assays as described below.

In Vitro Binding of Tiam1/Tiam1 Fragment to CD44—Aliquots (0.5–1 ng of protein) of purified FLAG-CD44cyt fusion protein bound to Anti-FLAG M2 antibody immunoaffinity beads were incubated in 0.5 ml of binding buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% bovine serum albumin, and 0.05% Triton X-100) containing various concentrations (10–800 ng/ml) of ^{125}I-labeled intact Tiam1 (purified from SP1 cells) (5000 cpm/ng protein) or ^{125}I-labeled recombinant Tiam1 fragment (CBP-tagged PHn-CC-Ex) at 4 °C for 4 h. Specifically, equilibrium binding conditions were determined by performing a time course (1–10 h) of ^{125}I-labeled Tiam1 (or CBP-tagged PHn-CC-Ex) binding to CD44 at 4 °C. The binding equilibrium was found to be established when the *in vitro* Tiam1 (or PHn-CC-Ex)-CD44 binding assay was conducted at 4 °C after 4 h. Following binding, the immunobeads were washed extensively in binding buffer, and the bead-bound radioactivity was counted. Nonspecific binding was determined using a 50–100-fold excess of unlabeled Tiam1 (or PHn-CC-Ex) in the presence of the same concentration of ^{125}I-labeled Tiam1 or ^{125}I-labeled CBP-tagged PHn-CC-Ex. Nonspecific binding, which was approximately 20% of the total binding, was always subtracted from the total binding. Our binding data are highly reproducible. The values expressed in Fig. 5 represent an average of triplicate determinations of three to five experiments with a standard deviation less than ± 5%.

In some cases, 0.1 μg of surface biotinylated CD44v3 was incubated with various Tiam1-related proteins (*e.g.* purified intact Tiam1, HA-tagged C1199, CBP-PHn-CC-Ex, or HA/CBP-coated beads) in the presence and absence of 100-fold excess amount of CBP-PHn-CC-Ex at room temperature in the binding buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% bovine serum albumin, and 0.05% Triton X-100) for 1 h. After binding, biotinylated CD44v3 bound to the beads was analyzed by SDS-polyacrylamide gel electrophoresis, transferred to the nitrocellulose filters, and incubated with ExtrAvidin-peroxidase (Sigma). After an addition of peroxidase substrate (Pierce), the blots were developed using ECL chemiluminescence reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Tiam1-mediated GDP/GTP Exchange for Rac1 Proteins—Purified *E. coli*-derived GST-tagged Rac1 (20pmol) was preloaded with GDP (30 μM) in 10 μl of buffer containing 25 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 4.7 mM EDTA, 0.16 mM $MgCl_2$, and 200 μg/ml bovine serum albumin at 37 °C for 7 min. To terminate preloading procedures, additional MgCl2 was then added to the solution (reaching a final concentration of 9.16 mM) as described previously (40, 49). Subsequently, 2 pmol of Tiam1 (in anti-Tiam1 (or anti-HA or anti-CD44v3)-Sepharose bead-conjugated forms) isolated from COS-7 cells (transfected with either the full-lengh Tiam1cDNA or NH_2-terminally truncated Tiam1cDNA) or SP1 cells (transfected with various plasmid DNAs such as HA-tagged C1199 Tiam1cDNA, GFP-tagged PHn-CC-ExcDNA or HA-tagged C1199Tiam1cDNA plus GFP-tagged PHn-CC-ExcDNA (as co-transfection) or vector alone, grown in the presence or absence of hyaluronic acid (100 μg/ml)) or control samples (nonspecific cellular material associated with preimmune serum-conjugated Sepharose beads) was preincubated with 2.5 μM [^{35}S]GTPγS (≈1,250 Ci/mmol) (in the presence or absence of 2.25 μM GTPγS for 10 min followed by adding 2.5 pmol of GDP-loaded GST-tagged Rac1GTPase as described previously) (49). The amount of [^{35}S]GTPγS bound to Tiam1 (conjugated to anti-Tiam1-Sepharose beads) or control sample (preimmune serum-conjugated Sepharose beads) in the absence of Rac1GTPase was subtracted from the original values. Data represent an average of triplicates from three to five experiments. The standard deviation was less than 5%.

Double Immunofluorescence Staining—SP1 cells (transfected with various plasmid DNAs such as HA-tagged C1199 Tiam1cDNA, GFP-tagged PHn-CC-ExcDNA, or HA-tagged C1199Tiam1cDNA plus GFP-tagged PHn-CC-ExcDNA (as co-transfection) or vector alone) were first washed with PBS buffer (0.1 M phosphate buffer (pH 7.5) and 150 mM NaCl) and fixed by 2% paraformaldehyde. Subsequently, SP1 transfectants were stained with rhodamine (Rh)-labeled rabbit anti-CD44v3 antibody. In some cases, Rh-labeled cells were then rendered permeable by ethanol treatment followed by incubating with FITC-conjugated mouse anti-HA IgG. To detect nonspecific antibody binding, Rh-CD44v3-labeled cells were incubated with FITC-conjugated normal mouse IgG. No labeling was observed in such control samples. The fluorescein- and rhodamine-labeled samples were examined with a confocal laser scanning microscope (MultiProbe 2001 Inverted CLSM system, Molecular Dynamics, Sunnyvale, CA).

Cell Adhesion Assay—SP1 cells were metabolically labeled with Tran^{35}S label (20 μCi/ml) as described above. After labeling, the cells were washed in PBS and incubated in PBS containing 5 mM EDTA at 37 °C to obtain a nonadherent single cell suspension. Labeled cells (≈9.1×10^5 cpm/10^5 cells) (in the presence or absence of anti-CD44v3 antibody) were plated on the HA-coated plates at 4 °C for 30 min. Following incubation, the wells were washed three times in PBS, the adherent cells were solubilized in PBS containing 1% SDS, and the well bound radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined by including 300 μg/ml soluble HA during the incubation of cells on HA-coated wells. The nonspecific binding was 10–15% of the total well-associated radioactivity and has been subtracted.

Tumor Cell Migration Assays—Twenty-four transwell units were used for monitoring *in vitro* cell migration as described previously (23). Specifically, the 5-μm porosity polycarbonate filters (CoStar Corp., Cambridge, MA) were used for the cell migration assay. SP1 cells (≈1×10^4 cells/well in PBS, pH 7.2) (in the presence or absence of anti-CD44v3 antibody (50 μg/ml)) were placed in the upper chamber of the transwell unit. In some cases, cells were transfected with either C1199Tiam1cDNA, PHn-CC-ExcDNA, C1199Tiam1cDNA plus PHn-CC-ExcDNA, or vector alone. The growth medium containing high glucose Dulbecco's modified Eagle's medium supplemented with 200 μg/ml hyaluronic acid was placed in the lower chamber of the transwell unit. After 18 h of incubation at 37 °C in a humidified 95% air/5% CO_2 atmosphere, the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Promega) was added at a final concentration of 0.2 mg/ml to both the upper and the lower chambers and incubated for an additional 4 h at 37 °C. Migrative cells at the lower part of the filter were removed by swabbing with small pieces of Whatman filter paper. Both the polycarbonate filter and the Whatman paper were placed in dimethyl sulfoxide to solubilize the crystal. Color intensity was measured in 450 nm. Cell migration processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters by standard cell number counting methods as described previously (23, 49). The CD44-specific cell migration was determined by subtracting nonspecific cell migration (*i.e.* cells migrate to the lower chamber in the presence of anti-CD44v3 antibody treatment) from the total migrative cells in the lower chamber. The CD44-specific cell migration in vector-transfected cells (control) is designated as 100%. Each assay was set up in triplicate

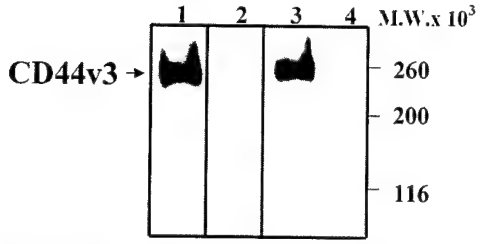


FIG. 1. **Expression of CD44v3 in breast tumor cells.** Breast tumor cells (SP1 cell line) were surface biotinylated (or unlabeled) and solubilized in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Triton X-100. The solubilized materials were then immunobloted or immunoprecipitated by anti-CD44v3 antibody as described under "Materials and Methods." *Lane 1*, immunoblot of unlabeled SP1 cell lysate using rabbit anti-CD44v3 antibody; *lane 2*, immunoblot of unlabeled SP1 cells with preimmune rabbit serum; *lane 3*, immunoprecipitation of surface biotinylated SP1 cells using rabbit anti-CD44v3 antibody; *lane 4*, immunoprecipitation of surface biotinylated SP1 cells with preimmune rabbit serum.

and repeated at least three times. All data were analyzed statistically using the Student's t test, and statistical significance was set at $p < 0.01$.

RESULTS

Identification of CD44 Variant Isoform(s) as HA Receptor(s) in SP1 Cells—The expression of CD44 variant isoforms such as CD44v3 is known to be closely correlated with metastatic and proliferative behavior of a variety of tumor cells including various carcinomas such as human breast tumor cells (14–19). Immunoblotting with anti-CD44v3 antibody (recognizing the v3-specific sequence located at the membrane-proximal region of the extracellular domain of CD44) indicates that a single CD44v3 protein (molecular mass = ~260 kDa) is expressed in SP1 cells (Fig. 1, *lane 1*). Furthermore, we have utilized surface biotinylation techniques and anti-CD44v3-mediated immunoprecipitation to characterize this CD44v3 molecule. Our results show that the 260-kDa CD44v3 molecule can be surface-biotinylated and is located on the surface of SP1 cells (Fig. 1, *lane 3*). No $CD44v_3$-containing material is observed in control samples when preimmune rabbit serum is used in either immunoblot (Fig. 1, *lane 2*) or immunoprecipitation experiments (Fig. 1, *lane 4*). Further analyses using reverse transcriptase-PCR, cloning, and nucleotide sequence techniques indicate that this CD44v3 belongs to the $CD44v_{3,8-10}$ isoform in SP1 cells (data not shown). This $CD44v_{3,8-10}$ variant exon structure was previously identified in human breast carcinoma samples (14–19), and its molecular mass (expressed at the protein level) has been shown to be ≈260 kDa (9).

CD44 is the major hyaluronan cell surface receptor (50), and a cellular adhesion molecule in many different cell types (51). Specific HA-binding motifs have been identified and localized in the extracellular domain of all CD44 isoforms (52, 53). To determine whether HA promotes cell adhesion, breast tumor cells (SP1 cell line) were incubated with plastic dishes coated with HA. As shown in Table I, SP1 cells adhere to the HA-coated dishes very well. In addition, because preincubation with anti-CD44v3 antibody blocks the adhesion of SP1 cells to HA-coated dishes, these data clearly indicate that CD44v3 isoform involves a specific binding interaction with the extracellular matrix component such as HA and is a cell surface adhesion molecule in SP1 cells.

Analysis of a Complex Formed between CD44v3 and Tiam1 in SP1 Cells in Vivo—Both CD44v isoforms (14–19) and Tiam1 (39) have been detected in a variety of tumor cells. In this study we have addressed the question of whether there is an interaction between CD44v3 isoform and Tiam1 in breast tumor cells (*e.g.* SP1 cells). First, we have analyzed Tiam1 expression

TABLE I
$CD44v_3$-mediated adhesion of metabolically labeled SP1 cells to HA-coated plates

Trans^{35}S-labeled SP1 cells were pretreated with or without anti-$CD44v_3$ antibody treatment. Subsequently, these cells were incubated in tissue culture wells coated with HA as described under "Materials and Methods." The background level of binding was determined by cell adhesion performed in the presence of an excess amount of soluble HA. The results were expressed in terms of HA-specific binding in which the background levels of binding have been subtracted. Data are expressed as mean cpm ± S.E. of triplicate determinations.

Treatments	$CD44v_3$-specific adhesion to HA-coated plates	
	cpm	*% of control*
Untreated cells (control)	5,057 ± 201	100
Anti-$CD44v_3$-treated cells	1,091 ± 136	21

(at the protein level) in breast tumor cells such as SP-1 cell line. Immunoblot analysis, utilizing anti-Tiam1 antibody designed to recognize the specific epitope located at the COOH-terminal sequence of Tiam1 reveals a single polypeptide (molecular mass = ~200 kDa) (Fig. 2, *lane 2*). We have demonstrated that Tiam1 detected in SP1 cells revealed by anti-Tiam1-mediated immunoblot is specific because no protein is detected in these cells using preimmune rabbit IgG (Fig. 2, *lane 1*). Furthermore, we have carried out anti-CD44v3-mediated and anti-Tiam1-mediated precipitation followed by anti-Tiam1 immunoblot (Fig. 2, *lane 3*) or anti-anti-CD44v3 immunoblot (Fig. 2, *lane 4*), respectively, using SDS-polyacrylamide gel electrophoresis analyses. Our results clearly indicate that the Tiam1 band is revealed in anti-CD44v3-immunoprecipitated materials (Fig. 2, *lane 3*). The CD44v3 band can also be detected in the anti-Tiam1-immunoprecipitated materials (Fig. 2, *lane 4*). These findings clearly establish the fact that CD44v3 and Tiam1 are closely associated with each other *in vivo* in breast tumor cells.

In Vitro Binding Between Tiam1 (or PHn-CC-Ex Domain) and CD44—Previous studies indicate that Tiam1 membrane localization (through its NH_2-terminal PHn domain and an adjacent protein interaction domain (designated as PHn-CC-Ex, a sequence between amino acids 393–738 of Tiam1)) (Fig. 3, *A* and *C*) is required for the activation of Rac1 signaling pathways leading to membrane ruffling and c-Jun NH_2-terminal kinase activation (37, 54). To test whether CD44 is one of the membrane proteins involved in the direct binding to Tiam1, we have used purified CBP-tagged PHn-CC-Ex fusion protein (Figs. 3*C* and 4, *lane 1*) and the FLAG-tagged cytoplasmic domain of CD44 (FLAG-CD44cyt) fusion protein (Fig. 4, *lane 2*) to identify the CD44 binding site on the Tiam1 molecule. Specifically, we have tested the binding of FLAG-CD44cyt to ^{125}I-labeled CBP-PHn-CC-EX (or ^{125}I-labeled intact Tiam1) under equilibrium binding conditions. Scatchard plot analyses presented in Fig. 5 indicate that PHn-CC-Ex binds to the cytoplasmic domain of CD44 (CD44cyt) at a single site (Fig. 5*A*) with high affinity (an apparent dissociation constant (K_d) of ≈0.2 nM). This interaction between PHn-CC-Ex and CD44 is comparable in affinity with CD44 binding (K_d = ~0.13 nM) to intact Tiam1 (Fig. 5*B*). These findings clearly indicate that Tiam1 (in particular, PHn-CC-Ex domain) contains the CD44 binding site.

Further analyses using an *in vitro* binding assay show that surface biotinylated CD44v3 (isolated from SP1) specifically binds to Tiam1 (including intact Tiam1 (Fig. 6, *lane 1*), HA-tagged C1199 Tiam1 (Fig. 6, *lane 2*) or Tiam1 fragment (PHn-CC-Ex) (Fig. 6, *lane 3*))-coated beads. In the presence of an excess amount (≈100-fold) of recombinant PHn-CC-Ex Tiam1 fragment, the binding interaction between CD44v3 and these-Tiam1-related proteins is readily abolished (Fig. 6, *lanes 4–6*). These observations suggest that (i) the breast tumor cell-spe-

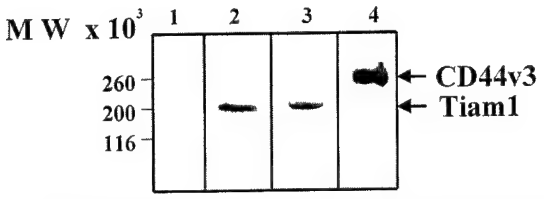


FIG. 2. **Detection of Tiam1 and Tiam1-CD44v3 complex in SP1 cells.** SP1 cells (5×10^5 cells) were solubilized by 1% Nonidet P-40 buffer followed by immunoprecipitation and/or immunblot by anti-Tiam1 antibody or anti-CD44v3 antibody, respectively, as described under "Materials and Methods." *Lane 1*, immunoblot of SP1 cells with preimmune rabbit serum; *lane 2*, detection of Tiam1 with anti-Tiam1-mediated immunoblot of SP1 cells; *lane 3*, detection of Tiam1 in the complex by anti-CD44v3-immunoprecipitation followed by immunoblotting with anti-Tiam1 antibody; *lane 4*, detection of CD44v3 in the complex by anti-Tiam1 immunoprecipitation followed by immunoblotting with anti-CD44v3 antibody.

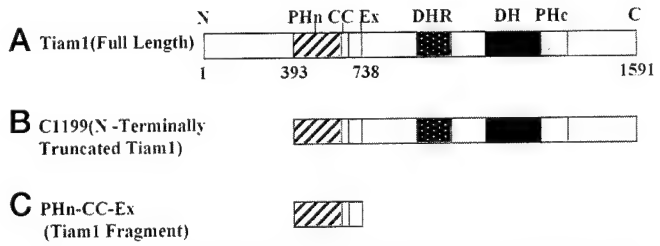


FIG. 3. **Illustration of Tiam1 full-length (*A*) and deletion mutant cDNA constructs (*B* and *C*).** The full-length Tiam1 contains a Dbl homology domain (*DH*), a Discs large homology region (*DHR*), two pleckstrin homology (*PH*) domains (including the NH_2-terminal PH (*PHn*) and the COOH-terminal PH (*PHc*)), a putative coiled coil region (*CC*), and an additional adjacent region (*Ex*). The NH_2-terminally truncated C1199 Tiam1 encodes the COOH-terminal 1199 amino acids (*B*). PHn-CC-Ex domain of Tiam1 encodes the sequence between amino acids 393 and 738 (*C*).

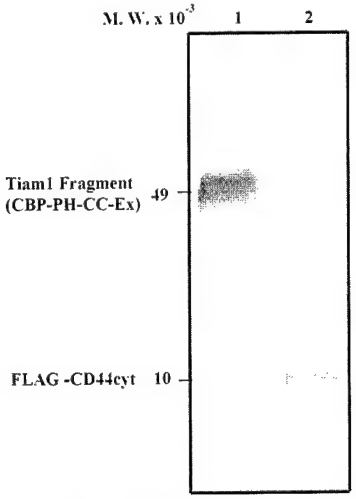
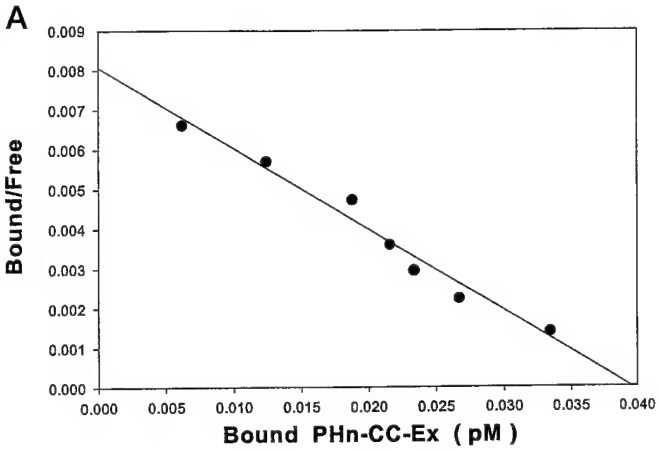


FIG. 4. **Characterization of various recombinant proteins used in the *in vitro* binding assay.** *Lane 1*, a Coomassie Blue staining of CBP-PH-CC-Ex fusion protein purified by calmodulin affinity resin column chromatography; *lane 2*, a Coomassie Blue staining of FLAG-CD44cyt fusion protein eluted from affinity column chromatography with FLAG peptide.



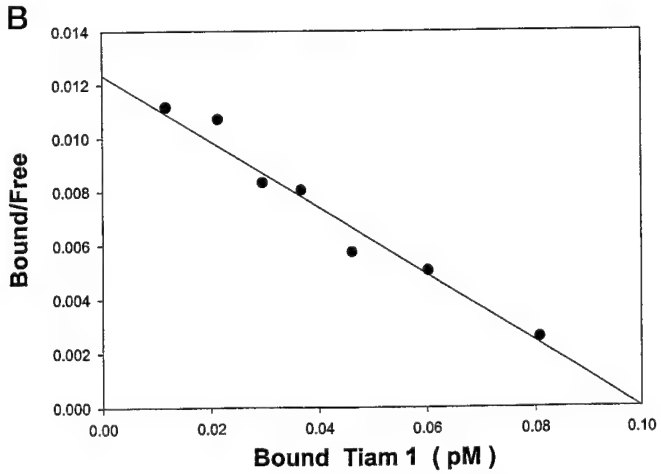


FIG. 5. **Binding of ^{125}I-labeled PHn-CC-Ex (or Tiam1) to FLAG-CD44cyt.** Various concentrations of ^{125}I-labeled PHn-CC-Ex (or Tiam1) were incubated with FLAG-CD44cyt-coupled beads at 4 °C for 4 h. Nonspecific binding was determined in the presence of 50-fold excess of unlabeled PHn-CC-Ex (or Tiam1) and subtracted from the total binding. Results represent an average of duplicate determinations from the same experiment. Data presented are the representative of three individual binding experiments. *A*, Scatchard plot analysis of the equilibrium binding data between ^{125}I-labeled PHn-CC-Ex and FLAG-CD44cyt. *B*, Scatchard plot analysis of the equilibrium binding data between ^{125}I-labeled intact Tiam1 and FLAG-CD44cyt.

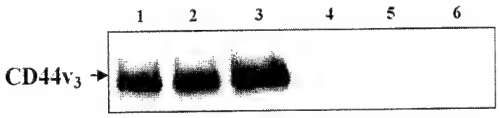


FIG. 6. ***In vitro* binding between CD44v3 and Tiam1-related protein.** CD44v3 was immunoprecipitated from surface biotinylated SP1 cells by anti-CD44v3 antibody as described under "Materials and Methods." Subsequently, purified surface biotinylated CD44v3 was incubated with Tiam1, C1199 Taim1, or PHn-CC-Ex-coated beads in the binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% bovine serum albumin, and 0.05% Triton X-100) at room temperature for 1 h. After extensive washing, protein bound on the beads were eluted and analyzed with Extravidin (horseradish peroxidase-conjugated). *Lane 1*, binding of CD44v3 to Tiam1-conjugated beads; *lane 2*, binding of CD44v3 to C1199 Tiam1-conjugated beads; *lane 3*, binding of CD44v3 to PHn-CC-Ex-conjugated beads; *lane 4*, binding of CD44v3 to Tiam1-conjugated beads in the presence of an excess amount (≈100-fold) of soluble PHn-CC-Ex; *lane 5*, binding of CD44v3 to C1199 Tiam1-conjugated beads in the presence of an excess amount (≈100-fold) of soluble PHn-CC-Ex; *lane 6*, binding of CD44v3 to PHn-CC-Ex-conjugated beads in the presence of an excess amount (≈100-fold) of soluble PHn-CC-Ex.

cific CD44v3 is also capable of interacting with Tiam1 (*e.g.* intact Tiam1 (Fig. 6, *lane 1*), HA-tagged C1199 Tiam1 (Fig. 6, *lane 2*), or Tiam1 fragment (PHn-CC-Ex) (Fig. 6, *lane 3*)); and (ii) the Tiam1 fragment such as PHn-CC-Ex acts as a potent competitive inhibitor for Tiam1 binding to CD44v3 *in vitro* (Fig. 6, *lanes 4–6*).

Tiam1-catalyzed Rac1 Activation in SP1 Cells—Rac1 GTPase becomes activated when bound GDP is exchanged for GTP by a process catalyzed by guanine nucleotide (GDP-GTP) exchange factors or GDP dissociation stimulator proteins (*i.e.* promoting GTP binding to RhoA by facilitating the release of GDP) (25,

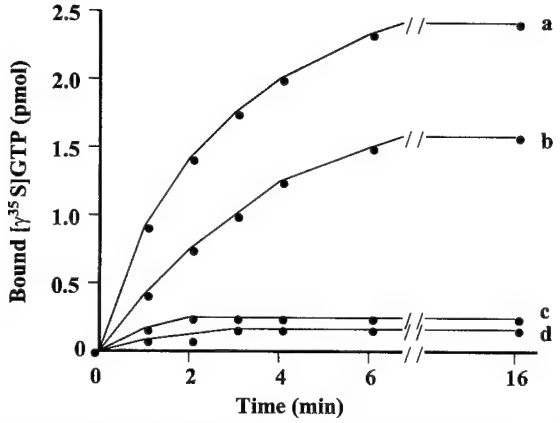


FIG. 7. **Tiam1-mediated GDP/GTP exchange for Rac1 protein.** Tiam1 isolated from SP1 cells (treated with HA or without any treatment) was preincubated for 10 min with 0.25 μM [^{35}S]GTPγS (1,250 Ci/mmol) and 2.25 μM GTPγS (or in the presence of 1 mM unlabeled GTPγS) followed by adding GDP-loaded GST-Rac1 GTPases (or GST alone). The amount of [^{35}S]GTPγS bound to samples in the absence of GTPases was subtracted from the original values. Data represent an average of triplicates from three to five experiments. The standard deviation was less than 5%. *Line a*, kinetics of [^{35}S]GTPγS bound to GDP-loaded GST-Rac1 in the presence of Tiam1 (isolated from SP1 cells treated with HA); *line b*, kinetics of GTPγ^{35}S bound to GDP-loaded GST-Rac1 in the presence of Tiam1 (isolated from SP1 cells without any treatment); *line c*, kinetics of [^{35}S]GTPγS bound to GDP-treated GST in the presence of Tiam1 (isolated from SP1 cells treated with HA); *line d*, kinetics of [^{35}S]GTPγS bound to GDP treated GST in the presence of Tiam1 (isolated from SP1 cells without any treatment).

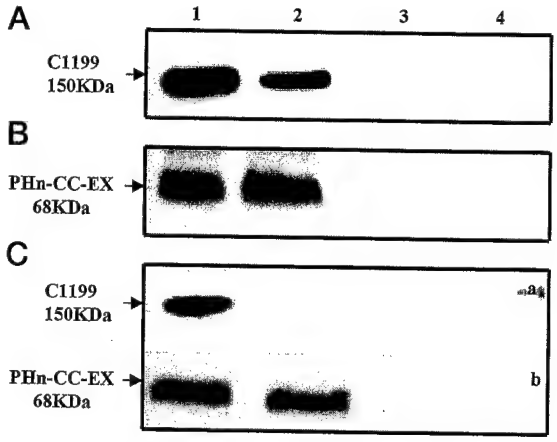


FIG. 8. **Transfection of SP1cells with HA-tagged C1199Tiam1cDNA (*A*) or GFP-tagged PHn-CC-ExcDNA (*B*) or co-transfection of HA-tagged C1199Tiam1cDNA and GFP-tagged PHn-CC-ExcDNA (*C*).** *A*, detection of C1199 Tiam1 expression by anti-HA-mediated immunoblot in HA-tagged C1199 Tiam1cDNA transfected cells (*lane 1*) or in vector-transfected cells (*lane 3*); immunoblot of anti-CD44v3 immunoprecipitated materials (*lane 2*) or rabbit preimmune IgG precipitated materials (*lane 4*) from HA-tagged C1199 Tiam1cDNA transfected cells with anti-HA antibody. *B*, detection of PHn-CC-Ex expression by anti-GFP-mediated immunoblot in GFP-tagged PHn-CC-Ex cDNA transfected cells (*lane 1*) or vector-transfected cells (*lane 3*); immunoblot of anti-CD44v3 immunoprecipitated materials (*lane 2*) or rabbit preimmune IgG precipitated materials (*lane 4*) from GFP-tagged PHn-CC-Ex cDNA transfected cells with anti-GFP antibody. *C*, detection of co-expression of C1199 Tiam1 and PHn-CC-Ex by immunoblotting of cells (co-transfected with HA-tagged C1199 Tiam1cDNA and GFP-tagged PHn-CC-Ex cDNA) with anti-HA antibody (*row a*, *lane 1*) and anti-GFP antibody (*row b*, *lane 1*), respectively; immunoblotting of vector-transfected cell lysate with anti-HA antibody (*row a*, *lane 3*) and anti-GFP antibody (*row b*, *lane 3*), respectively; immunoblot of anti-CD44v3 immunoprecipitated materials (from HA-tagged C1199 Tiam1cDNA and GFP-tagged PHn-CC-Ex cDNA co-transfected cells) using anti-HA antibody (*row a*, *lane 2*) and anti-GFP antibody (*row b*, *lane 2*), respectively. Immunoblot of rabbit preimmune IgG-precipitated materials (from HA-tagged C1199 Tiam1cDNA and GFP-tagged PHn-CC-Ex cDNA co-transfected cells) using anti-HA antibody (*row a*, *lane 4*) and anti-GFP antibody (*row b*, *lane 4*), respectively.

26). Tiam1 is known to function as an exchange factor for the Rho-like GTPases such as Rac1 (34, 40–42). To investigate whether Tiam1 complexed with CD44v3 acts as a GDP/GTP exchange factor (or a GDP dissociation stimulator protein) for *E. coli*-derived GST-Rac1, we have isolated Tiam1 complexed with CD44v3 from SP1 cells using anti-Tiam1-conjugated Sepharose beads. Our data show that Tiam1 complexed with CD44v3 from SP1 cells causes the exchange of preloaded GDP for [^{35}S]GTPγS on GST-Rac1 in a time-dependent manner (Fig. 7, *lines a* and *b*). Most importantly, addition of HA to CD44v$_3$ containing SP1 cells stimulates the total amount of bound [^{35}S]GTPγS to GST-Rac1 (Fig. 7, *line b*) (at least 1.5-fold increase) as compared with Tiam1 isolated from untreated SP1 cells (Fig. 7, *line b*) or HA-treated SP1 cells in the presence of anti-CD44v3 antibody (data not shown). No [^{35}S]GTPγS-bound material was detected in these samples containing GST alone under the same GDP/GTP exchange reaction using Tiam1 isolated from SP1 cells (in the presence (Fig. 7, *line c*) or absence (Fig. 7, *line d*) of HA treatment). These findings suggest that the HA interaction with CD44v$_3$ isoform-containing SP1 cells promotes Tiam1 activation of Rac1.

CD44v3-Tiam1 Interaction in Rac1 Signaling and Cytoskeleton-mediated Tumor Cell Migration—Previous studies indicate that the invasive phenotype of tumor cells characterized by an invadopodia structure (or membranous projections) (56, 57) and tumor cell migration (28, 29) is closely associated with CD44v$_{3,8-10}$-linked cytoskeleton function (23). In this study we have transiently transfected breast tumor cells (*e.g.* SP-1 cells) with HA-tagged NH$_2$-terminally truncated C1199 Tiam1 cDNA (Fig. 3*B*). Our results show that the C1199 Tiam1 is expressed as a 150-kDa protein (Fig. 8*A*, *lane 1*) detected by anti-HA-mediated immunoblot in CD44v$_3$-positive breast tumor cells (SP1 cells). No protein band was detected in vector-transfected SP1 cells by anti-HA-mediated immunoblotting (Fig. 8*A*, *lane 3*). Using anti-CD44v3 immunoprecipitation of SP1 cellular protein followed by immunoblotting with anti-HA antibody, we have found that the 150-kDa C1199 Tiam1 is co-precipitated with CD44v3 (Fig. 8*A*, *lane 2*). In control samples, immunoblotting of rabbit preimmune IgG-precipitated material using anti-HA antibody does not reveal any protein associated with this material (Fig. 8*A*, *lane 4*). Double immunofluorescence staining data also confirms the close association between CD44v3 (Fig. 9*A*) and the C1199 Taim1 (Fig. 9*B*) in the plasma membranes and long membrane projections. In contrast, vector-transfected cells expressing CD44v3 on the surface (Fig. 9, *inset a*) (with no detectable C1199 Tiam1 by anti-HA label (Fig. 9, *inset b*)) fail to display long membrane projections. Furthermore, we have demonstrated that transfection of SP1 cells with C1199 Tiam1 cDNA stimulates CD44v3-associated Tiam1-catalyzed GDP/GTP exchange on Rac1 (Table II) and induces a significant amount of increase in CD44v3-specific and HA-mediated breast tumor cell migration (Table II) compared with vector-transfected SP1 transfectants (Table II). These results are consistent with previous findings indicating that transfection of NIH3T3 cells with the NH$_2$-terminally truncated C1199 Tiam1 cDNA confers potent oncogenic properties (42). Treatment of SP1 cells (*e.g.* untransfected cells or transfected cells) with certain agents (*e.g.* cytochalasin D (a microfilament inhibitor)) causes a remarkable inhibition of CD44v3/HA-specific tumor cell migration (Table II). These observations suggest that CD44v3-associated Tiam1 signaling and cytoskeleton-mediated tumor cell motility are closely coupled.

Moreover, we have found that SP1 cells transfected with GFP-tagged PHn-CC-Ex Tiam1 cDNA express a 68-kDa pro-

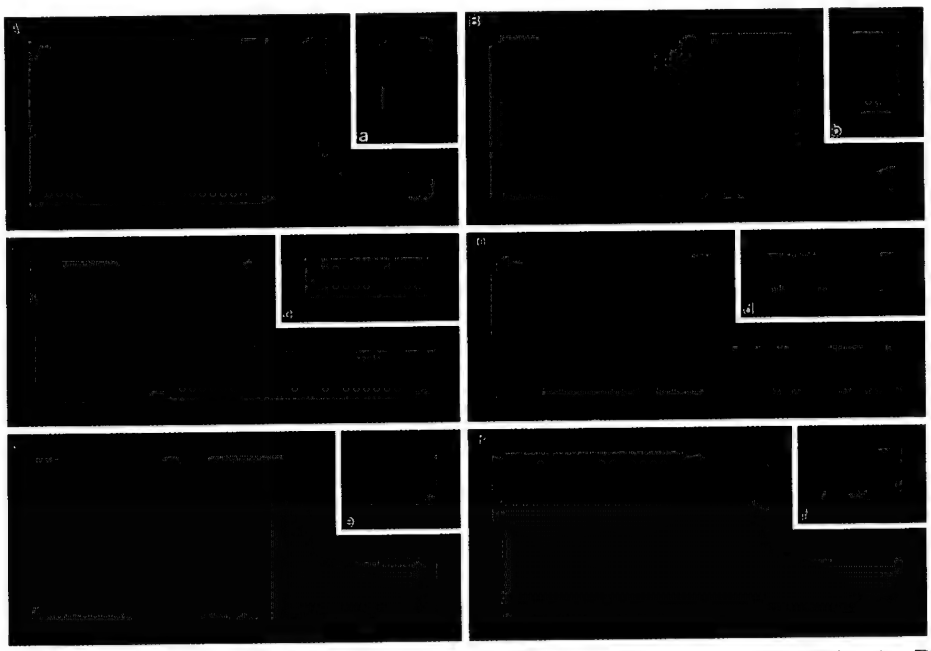


FIG. 9. **Double immunofluorescence staining of CD44v3 and Tiam1 cDNA (*e.g.* C1199 Tiam1 cDNA or PHn-CC-Ex cDNA)-transfected SP1 cells.** SP1 cells (transfected with HA-tagged C1199 Tiam1 cDNA or GFP-tagged PHn-CC-Ex cDNA or co-transfected with HA-tagged C1199 Tiam1 cDNA plus GFP-tagged PHn-CC-Ex cDNA) were fixed by 2% paraformaldehyde. Subsequently, cells were surface labeled with Rh-labeled rabbit anti-CD44v3 antibody. Some cells were then rendered permeable by ethanol treatment and stained with FITC-labeled mouse anti-HA IgG. *A* and *B*, Rh-labeled anti-CD44v3 staining (*A*) and FITC-anti-HA-labeled C1199 Tiam1 staining (*B*) in HA-tagged C1199 Tiam1 cDNA transfected SP1 cells. *Insets a* and *b*, Rh-labeled anti-CD44v3 staining (*a*) and FITC-anti-HA staining (*b*) in vector-transfected SP1 cells. *C* and *D*, Rh-labeled anti-CD44v3 staining (*C*) and GFP-tagged PHn-CC-Ex domain (*D*) in GFP-tagged PHn-CC-Ex cDNA transfected SP1 cells. *Insets c* and *d*, Rh-labeled preimmune IgG staining (*c*) and GFP-tagged PHn-CC-Ex domain (*d*) in GFP-tagged PHn-CC-Ex cDNA transfected SP1 cells. *E* and *F*, Rh-labeled anti-HA staining of C1199 Tiam1 (*E*) and GFP-tagged PHn-CC-Ex domain (*F*) in SP1 cells co-transfected with HA-tagged C1199 cDNA and GFP-tagged PHn-CC-Ex cDNA. *Insets e* and *f*, Rh-labeled anti-CD44v3 staining (*e*) and GFP-tagged PHn-CC-Ex domain (*f*) in SP1 cells co-transfected with HA-tagged C1199 cDNA and GFP-tagged PHn-CC-Ex cDNA.

tein as detected by anti-GFP antibody (Fig. 8*B*, *lane 1*). In vector-transfected SP1 cells, we are not able to detect any protein band by anti-GFP-mediated immunoblotting (Fig. 8*B*, *lane 3*). Using anti-CD44v3 immunoprecipitation of SP1 cellular protein followed by immunoblotting with anti-GFP antibody, we have found that the 68-kDa PHn-CC-Ex Tiam1 fragment is co-precipitated with CD44v3 (Fig. 8*B*, *lane 2*). No protein band was found when immunoblotting of rabbit preimmune IgG-precipitated materials with anti-GFP antibody was used (Fig. 8*B*, *lane 4*). It is also noted that both GFP-tagged PHn-CC-Ex domain (Fig. 9*D*) and CD44v3 are co-localized in the plasma membranes (Fig. 9*C*). However, no significant stimulation of long membrane projection was observed in these cells (Fig. 9, *C*, *D*, and *insets c* and *d*). Furthermore, we have demonstrated that CD44v3 staining detected in these SP1 transfectants revealed by anti-CD44v3-mediated immunostaining is specific because no surface label (Fig. 9, *inset c*) is detected in these GFP-PHn-CC-Ex overexpressed cells (Fig. 9, *inset d*) using preimmune rabbit IgG (Fig. 9, *inset c*). Additionally, we have demonstrated that overexpression of GFP-tagged PHn-CC-Ex domain in SP1 transfectants does not cause any significant changes of breast tumor cell properties (*e.g.* CD44v3-associated Tiam1-Rac1 signaling or HA-mediated tumor cell migration (Table II))

Finally, we have conducted co-transfection of SP1 cells with HA tagged C1199 Tiam1 cDNA and GFP-tagged PHn-CC-Ex cDNA. Our results indicate that C1199 Tiam1 and PHn-CC-Ex Tiam1 fragment are co-expressed as a 150-kDa protein (Fig. 8*C*, *row a*, *lane 1*) and a 68-kDa protein (Fig. 8*C*, *row b*, *lane 1*), respectively, in SP1 cells. No protein band was revealed in vector-transfected SP1 cells by anti-HA (Fig. 8*C*, *row a*, *lane 3*) or anti-GFP-mediated (Fig. 8*C*, *row b*, *lane 3*) immunoblotting. Using anti-C44v3 antibody immunoprecipitation of SP1 cell lysate followed by immunoblotting with anti-GFP antibody and anti-HA, respectively, we have found that the 68-kDa PHn-CC-Ex Tiam1 fragment (Fig. 8*C*, *row b*, *lane 2*) (but not 150-kDa C1199 Tiam1 (Fig. 8*C*, *row a*, *lane 2*)) is co-precipitated with CD44v3. In control samples, immunoblotting of rabbit preimmune IgG-precipitated material using anti-HA antibody (Fig. 8*C*, *row a*, *lane 4*) or anti-GFP antibody (Fig. 8*C*, *row b*, *lane 4*) does not reveal any protein associated with this material. Immunocytochemical staining results confirm that the PHn-CC-Ex Tiam1 fragment (Fig. 9, *inset e*) is co-localized with CD44v3 (Fig. 9, *inset f*) in the plasma membranes of SP1 transfectants. In contrast, the C1199 Tiam1 (Fig. 9*E*) fails to display plasma membrane localization as the PHn-CC-Ex domain does (Fig. 9*F*). Co-expression of PHn-CC-Ex domain and C1199 Tiam1 also efficiently blocks CD44v3-associated Tiam1-Rac1 activation and CD44v3-dependent and HA-mediated breast tumor cell migration (Table II). These results are consistent with a previous report showing that co-transfection of COS-7 cells with PHn-CC-Ex cDNA and C1199 Tiam1 cDNA results in an inhibition of C1199 Tiam1-induced Rac1 signaling and membrane ruffling (54). These findings suggest that the NH_2-terminal PHn domain and an adjacent protein interaction domain (PHn-CC-Ex) play an important role in regulating Tiam1 localization to the plasma membrane proteins such as CD44v3 isoforms and for oncogenic signaling during extracellular matrix component (*e.g.* hyaluronic acid)-regulated breast tumor cell invasion and migration.

DISCUSSION

CD44 denotes a family of glycoproteins (*e.g.* CD44s (standard form), CD44E (epithelial form), and CD44v (variant isoforms)) that are expressed in a variety of cells and tissues (1–6). Clinical studies indicate that a number of CD44v isoforms have been detected at high levels on the surface of tumor cells during tumorigenesis and metastasis (13–17). As the histologic grade

TABLE II

Measurement of CD44v_3-associated Tiam1-catalyzed Rac1 activation and HA-mediated/cytoskeleton-mediated breast tumor cell migration

	CD44v_3-associated Tiam1-catalyzed Rac1 activation: Amount of [γ-^{35}S]GTP bound to GST-Rac1[a]	
	pmol	*% of control*[b]
Untransfected cells (control)	1.50	100
Vector-transfected cells	1.47	98
PHn-CC-ExcDNA-transfected cells	1.42	95
C1199 Tiam1 cDNA-transfected cells	2.28	152
C1199 Tiam1 cDNA and PHn-CC-Ex cDNA co-transfected cells	1.35	90

	In vitro HA-mediated/CD44v_3-specific cell migration[a]	
	Me_2SO-treated control	Cytochalasin D-treated
	% of control[b]	
Untransfected cells (control)	100	20
Vector-transfected cells	97	25
PHn-CC-ExcDNA-transfected cells	95	23
C1199 Tiam1 cDNA-transfected cells	155	50
C1199 Tiam1 cDNA and PHn-CC-Ex cDNA co-transfected cells	90	15

[a] SP1 cells ($\approx 1 \times 10^4$ cells/well in PBS, pH 7.2) (in the presence or absence of 20 μg/ml cytochalasin D (dissolved in Me_2SO) or Me_2SO alone) were placed in the upper chamber of the transwell unit. In some cases, SP1 cells were transfected with either HA-tagged C1199 Tiam1 cDNA or GFP-tagged PHn-CC-ExcDNA or HA-tagged C1199 Tiam1 cDNA plus GFP-tagged PHn-CC-ExcDNA or vector alone. After 18 h of incubation at 37 °C in a humidified 95% air/5% CO_2 atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swab. Cell migration processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters containing HA by standard cell number counting assays as described under "Materials and Methods." Procedures for measuring CD44v_3-associated Tiam1-catalyzed GDP/GTP exchange reaction on GST-Rac1 were described under "Materials and Methods." Each assay was set up in triplicate and repeated at least three times. All data were analyzed statistically by Student's *t* test, and statistical significance was set at $p < 0.01$. In these experiments $\approx$30–40% of input cells ($\approx 1 \times 10^4$ cells/well) undergo *in vitro* cell invasion and migration in the control samples.

[b] The values expressed in this table represent an average of triplicate determinations of three to five experiments with a standard deviation of less than 5%.

of each of the tumors progresses, the percentage of lesions expressing an associated CD44v isoform increases. In particular, the CD44v3-containing isoforms are detected preferentially on highly malignant breast carcinoma tissue samples. In fact, there is a direct correlation between CD44v3 isoform expression and increased histologic grade of the malignancy (14, 17, 57).

It has been speculated that some of these CD44v3 isoforms on epithelial cells may act as surface modulators to facilitate unwanted growth factor receptor-growth factor interactions (9, 10) and subsequent tumor formation. The CD44-related glycoproteins are also known to mediate cell adhesion to extracellular matrix components (*e.g.* HA) and to function as the major hyaluronate receptor (50). In this study we have demonstrated that a 260-kDa CD44v3 isoform is expressed on the surface of breast tumor cells (SP1 cell line) (Fig. 1) and that it interacts with extracellular matrix HA as an adhesion receptor (Table I). Furthermore, addition of HA to SP1 cells stimulates tumor cell migration in a CD44v3-specific and cytoskeleton-dependent manner (Table II). These findings are consistent with previous findings that CD44v isoforms expressed in tumor cells often display enhanced hyaluronic acid binding, which increases cell migration capability (58, 59).

The invasive phenotype of CD44v3-mediated breast tumor cells, characterized by invadopodia formation (23), matrix metalloproteinase-9 activation (23, 24), and tumor cell motility (23, 48) has been linked to cytoskeletal function, a process in which the small GTP-binding proteins such as RhoA and Rac1 are shown to play important roles. Tsukita and co-workers (60) have reported that Rho-like proteins participate in the interaction between the CD44 and the ERM cytoskeletal proteins. Our recent study determined that RhoA is physically linked to CD44v3 isoform (*e.g.* CD44$v_{3,\ 8-10}$) in breast tumor cells (48). Rho-kinase stimulated by activated RhoA (GTP-bound form of RhoA) appears to play a pivotal role in promoting CD44$v_{3,8-10}$-ankyrin interaction during membrane-cytoskeleton function and metastatic breast tumor cell migration (48). Signaling to the RacGTPase known to regulate actin assembly associated with membrane ruffling, pseudopod extension, cell motility, and cell transformation (33–37) has been shown to be abnormal in breast tumor cells as compared with normal breast epithelial cells (61). The fact that Rac1 induces stress fiber formation in a Rho-dependent manner suggests that cross-talk occurs between the Rho and Rac1 signaling pathways (33). The question of whether the activation of Rac1 signaling is involved in CD44v3-cytoskeleton-mediated breast tumor-specific events remains to be answered.

Tiam1, which was identified by retroviral insertional mutagenesis and selected for its invasive cell behavior *in vitro*, has been shown to regulate Rac1 activation (38, 39). This molecule is largely hydrophilic and contains several functional domains including a Dbl homology domain (38, 62, 63), a Discs large homology region (38, 64), and two pleckstrin homology (PH) domains (*e.g.* PHn (the PH domain located at the NH_2-terminal region of the molecule; and PHc (the PH domain located at the COOH-terminal region of the molecule)) (Fig. 3) (38). In particular, the Dbl homology domain of Tiam1 exhibits GDP/GTP exchange activity for specific members of the Ras superfamily of GTP-binding proteins (62, 63) and plays an important role in Rac1 signaling and cellular transformation (33–37). In breast tumor cells (*e.g.* SP1 cells), Tiam1 is detected as a 200-kDa protein (Fig. 2) that is capable of carrying out GDP/GTP exchange for Rac1 (Fig. 7), similar to Tiam1 described in other cell types (34, 40–42, 65, 66). Other functional domains such as Discs large homology region have been implicated in the binding of membrane protein networks (38, 64). The PH domain may mediate association with the submembrane region of the cell via protein-protein or protein-lipid interactions (67). Based on mutational analyses and immunofluorescence staining, Collard and co-workers (37, 54) report that the NH_2-terminal PHn domain (but not PHc) and an adjacent protein interaction domain (*e.g.* PHn-CC-Ex domain) (Fig. 3) are required for Tiam1 targeting to the plasma membrane and Rac1 activation in fibroblasts. At the present time, identification of the membrane protein(s) involved in Tiam1 binding has not been established.

In this study we have presented new evidence that a close interaction occurs between Tiam1 and certain plasma membrane proteins such as CD44v3 isoform. Using two recombinant proteins (CBP-tagged PHn-CC-Ex domain (Fig. 4, *lane 1*) and FLAG-tagged CD44 cytoplasmic domain (FLAG-CD44cyt) (Fig. 4, *lane 2*)), we have demonstrated that the PHn-CC-Ex domain of Tiam1 is directly involved in the binding to the cytoplasmic domain of CD44 (Figs. 5 and 6). In fact, the binding affinity of the PHn-CC-Ex domain of Tiam1 to CD44 is comparable with the intact Tiam1 binding to CD44 (Figs. 5 and 6). In the presence of PHn-CC-Ex, the binding between Tiam1 and CD44 (*e.g.* CD44v3) is greatly reduced (Fig. 6). The ability of PHn-CC-Ex to effectively compete for Tiam1 binding to the plasma membrane proteins such as CD44v3 (Fig. 6) strongly suggests that the PHn-CC-Ex of Tiam1 is responsible for the recognition of CD44 *in vitro*.

In addition, we have detected that Taim1 and CD44v3 are physically linked to each other as a complex *in vivo* (Figs. 2, 8,

and 9) and that HA binding to CD44v3 promotes Tiam1-catalyzed Rac1 activation (Fig. 7 and Table II) and tumor cell migration (Table II). Our data also indicate that overexpression of Tiam1 (by transfecting SP1 cells with C1199 Tiam1cDNA) (Figs. 8 and 9) not only promotes C1199 Tiam1 association with CD44v3 (Figs. 8 and 9) but also enhances the metastatic capability of tumor cells (*e.g.* Rac1 activation and tumor cell migration (Table II)). These results suggest that Tiam1 and CD44v3 are not only structurally linked but also functionally coupled. Previously, it has been shown that Tiam1-activated Rac1 initiates oncogenic signaling cascades that involve activation of c-Jun NH_2-terminal kinase (37, 54) and a novel family of serine/threonine kinases, Paks (p-21 activated kinases) (68, 69). However, the identification of CD44v3-Tiam1-mediated downstream targets (*e.g.* c-Jun NH_2-terminal kinase and/or Paks activities) during HA-mediated breast tumor progression and metastasis remains to be answered.

Furthermore, we have found that co-transfection of SP1 cells with PHn-CC-Ex cDNA and C1199 Tiam1cDNA (Figs. 8 and 9) effectively blocks tumor cell-specific behaviors (*e.g.* C1199 Tiam1 association with CD44v3 (Figs. 8 and 9), Rac1 signaling (Table II), and tumor cell migration (Table II)). These findings further support our conclusion that PHn-CC-Ex acts as a potent competitive inhibitor that is capable of interfering with C1199 Tiam1-CD44v3 interaction *in vivo*. Recently, we have also identified a unique sequence residing within the PHn-CC-Ex domain as the putative cytoskeletal binding site of Tiam1 (70). Most importantly, interaction between Tiam1 and the cytoskeleton up-regulates the GDP/GTP exchange activity of Rho-like GTPases and stimulates breast tumor cell invasion/migration (70). These observations clearly suggest that the PHn-CC-Ex fragment of Tiam1 is one of the important regulatory domains required for Tiam1 function.

In fibroblasts, Tiam1-induced membrane ruffling is dependent on Rac1 (but not RhoA) activity (71). The fact that Tiam1 is involved in both Rac1- and RhoA-mediated pathways during neurite formation in nerve cells suggests that the balance between two Tiam1-activated Rho-like GTPases (*e.g.* Rac1 and RhoA) determines a particular biological activity (65). Tiam1-Rac1 signaling is also implicated in promoting integrin-mediated cell-cell and cell-extracellular matrix interaction and lymphoid cell invasion (34, 65). In addition, the laminin receptor, $\alpha_6\beta_1$ integrin appears to require Rac1 as a downstream of Tiam1 signaling in neuroblastoma cell activation (65). In epithelial Madin-Darby canine kidney cells, fibronectin and/or laminin1-induced Tiam1-Rac1 signaling up-regulates E-cadherin-mediated adhesion and plays an invasion-suppressor role in Ras-transformed Madin-Darby canine kidney cells (66). However, if Madin-Darby canine kidney cells were grown on different collagen substrates, the expression of Tiam1 or constitutively activated Rac1 (V12Rac) in these cells is able to inhibit the appearance of E-cadherin adhesion and promote cell migration (72, 73). Our studies show that approximately 60% ($63 \pm 4\%$, $n = 5$) of the GDP dissociation activity can be detected in the guanine nucleotide exchange assay using Tiam1 isolated from untreated breast tumor cells (Fig. 7 and Table II). We have also observed that approximately 90% ($92 \pm 5\%$, $n = 5$) of Rac1 is exchanging GDP for GTP in the presence of Tiam1 isolated from either HA-treated cells (Fig. 7) or C1199 Tiam1 cDNA-transfected breast tumor cells (Table II). These findings suggest that Tiam1-catalyzed Rac1 activation is tightly regulated by various signals. Apparently, different responses by Tiam1-catalyzed Rho-like GTPases are controlled by specific upstream activators (in particular, cell adhesion receptors (*e.g.* CD44, integrin, or E-cadherin, etc.) or extracellular matrix components (*e.g.* HA, collagens, laminin, or fibronectin, etc.)), which may result in selective Tiam1-activated Rho-like GTPases and distinct biological outcome. In summary, we believe that Tiam1-CD44v3 interaction plays a pivotal role in regulating oncogenic signaling required for RhoGTPase activation and cytoskeleton function during HA-mediated metastatic breast tumor cell progression. This could be one of the critical steps in CD44 variant isoform-mediated breast tumor spread and metastasis.

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Ankyrin–Tiam1 Interaction Promotes Rac1 Signaling and Metastatic Breast Tumor Cell Invasion and Migration

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Abstract. Tiam1 (T-lymphoma invasion and metastasis 1) is one of the known guanine nucleotide (GDP/GTP) exchange factors (GEFs) for Rho GTPases (e.g., Rac1) and is expressed in breast tumor cells (e.g., SP-1 cell line). Immunoprecipitation and immunoblot analyses indicate that Tiam1 and the cytoskeletal protein, ankyrin, are physically associated as a complex in vivo. In particular, the ankyrin repeat domain (ARD) of ankyrin is responsible for Tiam1 binding. Biochemical studies and deletion mutation analyses indicate that the 11–amino acid sequence between amino acids 717 and 727 of Tiam1 (^{717}GEGTDAVKRS^{727}L) is the ankyrin-binding domain. Most importantly, ankyrin binding to Tiam1 activates GDP/GTP exchange on Rho GTPases (e.g., Rac1).

Using an *Escherichia coli*–derived calmodulin-binding peptide (CBP)–tagged recombinant Tiam1 (amino acids 393–728) fragment that contains the ankyrin-binding domain, we have detected a specific binding interaction between the Tiam1 (amino acids 393–738) fragment and ankyrin in vitro. This Tiam1 fragment also acts as a potent competitive inhibitor for Tiam1 binding to ankyrin. Transfection of SP-1 cell with Tiam1 cDNAs stimulates all of the following: (1) Tiam1–ankyrin association in the membrane projection; (2) Rac1 activation; and (3) breast tumor cell invasion and migration. Cotransfection of SP1 cells with green fluorescent protein (GFP)–tagged Tiam1 fragment cDNA and Tiam1 cDNA effectively blocks Tiam1–ankyrin colocalization in the cell membrane, and inhibits GDP/GTP exchange on Rac1 by ankyrin-associated Tiam1 and tumor-specific phenotypes. These findings suggest that ankyrin–Tiam1 interaction plays a pivotal role in regulating Rac1 signaling and cytoskeleton function required for oncogenic signaling and metastatic breast tumor cell progression.

Key words: Tiam1 • ankyrin • Rac1 signaling • invasion/migration • metastatic breast tumor cells

Introduction

Members of the Rho subclass of the ras superfamily (small molecular masses GTPases, e.g., Rac1, RhoA, and Cdc42) are known to be associated with changes in the membrane-linked cytoskeleton (Ridley and Hall, 1992; Hall, 1998). For example, activation of Rac1, RhoA, and Cdc42 has been shown to produce specific structural changes in the plasma membrane cytoskeleton associated with membrane ruffling, lamellipodia, filopodia, and stress fiber formation (Ridley and Hall, 1992; Hall, 1998). The coordinated activation of these GTPases is thought to be a possible mechanism underlying cell motility, an obvious prerequisite for metastasis (Jiang et al., 1994; Dickson and Lippman, 1995; Lauffenburger and Horwitz, 1996).

Several guanine nucleotide exchange factors (GEFs,[1] the dbl or DH family) have been identified as oncogenes because of their ability to upregulate Rho GTPase activity during malignant transformation (Van Aelst and D'Souza-Schorey, 1997). One of these GEFs is Tiam1 (T-lymphoma invasion and metastasis 1), which was identified by retroviral insertional mutagenesis and selected for its invasive cell behavior in vitro (Habets et al., 1994, 1995). This molecule is largely hydrophilic and contains several functional domains found in signal transduction proteins. For example, the COOH-terminal region of the Tiam1 molecule has a Dbl homology (DH) domain (Hart et al., 1991, 1994; Habets et al., 1994) and an adjacent pleckstrin homology

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[1]*Abbreviations used in this paper:* ARD, ankyrin repeat domain; CBP, calmodulin-binding peptide; DH, Dbl homology; GFP, green fluorescent protein; GFP-SBD, GFP-tagged spectrin binding domain; GEF, guanine nucleotide exchange factor; HA, hemagglutinin; PH, pleckstrin homology; PHn, NH_2-terminal PH; Rh, rhodamine; S1P, sphingosine-1-phosphate; SBD, spectrin binding domain; Tiam1, T lymphoma invasion and metastasis 1.

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(PH) domain, which exists in most GEFs (Hart et al., 1991, 1994; Habets et al., 1994; Lemmon et al., 1996). In particular, the DH domain of these proteins exhibits GDP/GTP exchange activity for specific members of the Ras superfamily of GTP-binding proteins (Hart et al., 1991, 1994). Tiam1 also contains an additional PH domain, a Discs-large homology region (DHR; Habets et al., 1994; Pontings and Phillips, 1995), and a potential myristoylation site in the NH_2-terminal part of the protein (Habets et al., 1994).

Overexpression of both NH_2 and COOH terminally truncated as well as full-length Tiam1 proteins induces the invasive phenotype in otherwise noninvasive lymphoma cell lines (Michiels et al., 1995). It is also well established that Tiam1 is capable of activating Rac1 in vitro as a GEF, and induces membrane cytoskeleton–mediated cell shape changes, cell adhesion, and cell motility (Woods and Bryant 1991; Michiels et al., 1995; Nobes and Hall, 1995; Van Leeuwen et al., 1995). These findings have prompted investigations into the mechanisms involved in the regulation of Tiam1. In fact, it has been found that addition of certain serum-derived lipids (e.g., sphingosine-1-phosphate [S1P] and LPA) to T-lymphoma cells promotes Tiam1-mediated Rac1 signaling and T-lymphoma cell invasion (Stam et al., 1998). A Tiam1 transcript has been detected in breast cancer cells (Habets et al., 1995). Tiam1 is shown to function as a GEF in activating Rac1 signaling in breast tumor cells (Bourguignon et al., 2000). The question of how this molecule is regulated in invasive and metastatic processes of breast cancer cells is addressed in the present study.

Ankyrin belongs to a family of cytoskeletal proteins that mediate linkage of integral membrane proteins with the spectrin-based skeleton in regulating a variety of biological activities (Bennett, 1992; Bennett and Gilligan, 1993; De Matteis and Morrow, 1998). Presently, at least three ankyrin genes have been identified: *ankyrin 1* (ANK1 or *ankyrin R*), *ankyrin 2* (ANK2 or ankyrin B), and *ankyrin 3* (ANK3 or *ankyrin G*) (Lambert et al., 1990; Lux et al., 1990; Tse et al., 1991; Otto et al., 1991; Peters and Lux 1993; Kordeli et al., 1995; Peters et al., 1995). All ankyrin species (e.g., ANK1, ANK2, and ANK3) are monomers comprised of two highly conserved domains and a variable domain. Both conserved domains are located in the NH_2-terminal region and include a membrane-binding site (~89–95 kD, also called the ankyrin repeat domain [ARD]; Davis and Bennet, 1990; Lux et al., 1990), and a spectrin binding domain (SBD, ~62 kD; Platt et al., 1993). The striking feature shared by all three forms of ankyrins is the repeated 33–amino acid motif present in 24 contiguous copies within the ARD. The ARD of ANK1, ANK2, and ANK3 is highly conserved. A number of tumor cells express ankyrin such as ANK1 and ANK3 (Bourguignon et al., 1998a,b; Zhu and Bourguignon, 2000). Most recently, we have found that ankyrin's ARD interacts with the adhesion molecule, CD44, and promotes tumor cell migration (Zhu and Bourguignon, 2000). In addition, the ARD domain (also referred to as cdc 10 repeats, cdc10/SW16 repeats, and SW16/ANK repeats) has been detected in a number of functionally distinct proteins participating in protein–protein binding and protein–DNA interactions (Davis and Bennett, 1990; Lux et al., 1990).

In this study, we have focused on the regulatory aspect of Tiam1-Rac1 signaling in metastatic breast tumor cells (SP-1 cell line). Our results indicate that Tiam1 interacts with ankyrin in vivo and in vitro. In particular, the ankyrin repeat domain (ARD) is directly involved in Tiam1 binding. Biochemical analyses show that the Tiam1 fragment (amino acids 393–738) contains an ankyrin-binding site and competes for Tiam1 binding to ankyrin. Most importantly, the binding of ankyrin, in particular, the ankyrin repeat domain (ARD), to Tiam1 activates Rho-like GTPases such as Rac1. Overexpression of Tiam1 in SP-1 cells by transfecting Tiam1 cDNA induces Tiam1–ankyrin association in the cell membrane, Rac1 signaling, and metastatic phenotypes. Both Tiam1–ankyrin interaction and tumor-specific behaviors are significantly inhibited by cotransfecting SP-1 cells with the Tiam1 (amino acids 393–738) fragment cDNA and Tiam1 cDNA. Our observations suggest that Tiam1 interaction with ankyrin promotes Rho GTPase activation and cytoskeletal changes required for metastatic breast tumor cell invasion and migration.

Materials and Methods

Cell Culture

Mouse breast tumor cells (e.g., SP1 cell line; provided by Dr. Bruce Elliott, Department of Pathology and Biochemistry, Queen's University, Kingston, Ontario, Canada) were used in this study. Specifically, the SP1 cell line was derived from a spontaneous intraductal mammary adenocarcinoma that arose in a retired female CBA/J breeder in the Queen's University animal colony. These cells were capable of inducing lung metastases by sequential passage of SP1 cells into mammary gland (Elliott et al., 1988). These cells were cultured in RPMI 1640 medium supplemented with either 5 or 20% FCS, folic acid (290 mg/l), and sodium pyruvate (100 mg/l). COS-7 cells were obtained from American Type Culture Collection and grown routinely in DME containing 10% FBS, 1% glutamine, 1% penicillin, and 1% streptomycin.

Antibodies and Reagents

For the preparation of polyclonal rabbit anti-Tiam1 antibody, specific synthetic peptides (~15–17 amino acids unique for the COOH-terminal sequence of Tiam1) were prepared by the Peptide Laboratories of the Department of Biochemistry and Molecular Biology using an automatic synthesizer (model ACT350; Advanced Chemtech). These Tiam1-related polypeptides were conjugated to polylysine and subsequently injected into rabbits to raise the antibodies. The anti-Tiam1–specific antibody was collected from each bleed and stored at 4°C containing 0.1% azide. The anti-Tiam1 IgG fraction was prepared by conventional DEAE-cellulose chromatography. Mouse monoclonal anti-hemagglutinin (HA epitope) antibody (clone 12 CA5) and mouse monoclonal anti–green fluorescent protein (GFP) antibody were purchased from Boehringer Mannheim and PharMingen, respectively. *Escherichia coli* (*E. coli*)–derived GST-tagged Rac1/Cdc42 and GST-tagged RhoA was provided by Dr. Richard A. Cerione (Cornell University, Ithaca, NY) and Dr. Martin Schwartz (Scripps Research Institute, La Jolla, CA), respectively. Mouse monoclonal erythrocyte ankyrin (ANK1) and ANK3 antibodies were prepared as described previously (Bourguignon et al., 1993a). Rabbit anti-ANK3 antibody was provided by Dr. L.L. Peters (Jackson Laboratory, Bar Harbor, ME; Peters et al., 1995).

Cloning, Expression, and Purification of GST-tagged Ankyrin Repeat Domain (GST-ARD) and GFP-tagged Spectrin Binding Domain (GFP-SBD) of Ankyrin

pGEX-2TK recombinant plasmid expressing GST-ARD (NH_2-terminal portion of ankyrin, residues 1–834) was constructed as follows. Two pGEX-2TK recombinant plasmids pA3-79 (expressing epithelial Ank3 NH_2-terminal 1–455 amino acids) and pA3-88 (expressing epithelial Ank3

NH_2-terminal 317–834 amino acids; Peters et al., 1995) were provided by Dr. L.L. Peters from the Jackson Laboratory. The two plasmids were digested by EcoRI (one of pGEX-2TK vector cloning sites) and NheI (in ankyrin cDNA 1,176 bp) sequentially. The digested products were run in 1% agarose gel and purified with a purification kit (QIAGEN). The larger cDNA fragment in pA3-79–digested products (containing the pGEX-2TK vector and ankyrin cDNA 1–1,176 bp) and the smaller one in pA3-88–digested products (containing ankyrin cDNA 1,176–2,556 bp) were cut and purified. These two cDNA fragments were ligated and transformed to INVαF′-competent cells. The obtained clones were sequenced to verify the correct generation of the full-length ARD.

Spectrin binding domain (SBD) cDNA of human erythrocyte ankyrin was cloned into the eukaryotic expression vector, GFPN1 (CLONTECH Laboratories, Inc.) using the PCR-based cloning strategy. Ankyrin's SBD cDNA was amplified by PCR with two specific primers (left, 5′-CGCTCGAGATGAAGGCTGAGAGGCGGGATTCC-3′ and right, 5′-ATAAGCTTCAGGGGCGTCGGGGTCCTTCT-3′) linked with specific enzyme digestion site (XhoI and HindIII). The PCR product, which was digested with XhoI and HindIII, was purified with QIAquick PCR purification kit (QIAGEN). Ankyrin's SBD cDNA fragment was cloned into GFPN1 vector digested with XhoI and HindIII. The cDNA sequence was confirmed by nucleotide sequencing analysis. The GFP-tagged spectrin binding domain (GFP-SBD) of ankyrin is expressed as an 89-kD polypeptide in SP1 or COS-7 cells by SDS-PAGE and immunoblot analyses. The 89-kD GFP-SBD (but not ARD) displays specific spectrin binding property as described previously (Platt et al., 1993). Subsequently, GFP-SBD was isolated from anti-GFP–conjugated affinity columns and used in various in vitro binding experiments as described below.

Expression Constructs

Both the full-length mouse Tiam1 cDNA (FL1591) and the NH_2 terminally truncated Tiam1 cDNA (C1199) were provided by Dr. John G. Collard (The Netherlands Cancer Institute, The Netherlands). Specifically, the full-length Tiam1 (FL1591) cDNA was cloned into the eukaryotic expression vector, pMT2SM. The truncated C1199 Tiam1 cDNA (carrying a hemagglutinin epitope [HA] tag at the 3′ end) was cloned in the eukaryotic expression vector, pUTSV1 (Eurogentec, Belgium).

The deletion construct, HA-tagged C1199 Tiam1Δ717-727 (deleting the sequence between amino acids 717 and 727 of Tiam1) was derived from C1199 Tiam1 using QuickChange™ site-directed mutagenesis kit (Stratagene). In brief, two complimentary mutagenic oligonucleotide primers containing the desired deletion (5′-CCCAACCATCAACCAGGTGTTTGAGGGAATATTTGATG-3′) was designed and synthesized. First, the cycling reaction, using 30-ng double-stranded DNA template of C1199 Tiam1 plasmid and two complimentary primers, was performed to produce mutated cDNA according to the manufacturer's instruction. Subsequently, 1 μl of the DpnI restriction enzyme (10 U/μl) was added directly to the cycling reaction products to digest the parental supercoiled double-stranded DNA. This DpnI-treated cDNA was used to transform supercompetent cells (e.g., *Epicurian coli* XL 1-blue). Finally, the deletion construct was confirmed by DNA sequencing.

The Tiam1 (amino acids 393–728) fragment was cloned into calmodulin-binding peptide (CBP)–tagged vector (pCAL-n; Stratagene) using the PCR-based cloning strategy. Using human Tiam1 cDNA as a template, the Tiam1 fragment was amplified by PCR with two specific primers (left, 5′-AACTCGAGATGAGTACCACCAACAGTGAG-3′ and right, 5′-AAAAAGCTTTCAGCCATCTGGAACAGTGTCATC-3′) linked with a specific enzyme digestion site (XhoI or HindIII). The PCR product, which was digested with XhoI and HindIII, was purified with QIAquick PCR purification kit (QIAGEN). The Tiam1 fragment cDNA was cloned into pCAL-n vector digested with XhoI and HindIII. The inserted Tiam1 fragment sequence was confirmed by nucleotide sequencing analyses. The recombinant plasmids were transformed to BL21-DE3 to produce CBP-tagged Tiam1 fragment fusion protein. This fusion protein was purified from bacteria lysate by calmodulin affinity resin column (Sigma Chemical Co.).

The Tiam1 fragment cDNA was also cloned into pEGFPN1 vector (CLONTECH Laboratories, Inc.) digested with XhoI and HindIII to create GFP-tagged Tiam1 fragment cDNA. The inserted Tiam1 fragment sequence was confirmed by nucleotide sequencing analyses. This GFP-tagged Tiam1 fragment cDNA was used for transient expression in SP1 cells as described below. The GFP-tagged Tiam1 fragment is expressed as a 68-kD polypeptide in SP1 or COS-7 cells by SDS-PAGE and immunoblot analyses.

Cell Transfection

To establish a transient expression system, cells (e.g., SP-1 or COS-7 cells) were transfected with various plasmid DNAs including Tiam1 cDNAs (e.g., the full-length mouse Tiam 1 cDNA [FL1591], or HA-tagged C1199 Tiam1 cDNA, or HA-tagged C1199 Tiam1Δ717-727 cDNA, or GFP-tagged Tiam1 fragment cDNA, or HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA (cotransfection), or vector control constructs) using electroporation methods. In brief, cells (e.g., SP-1 or COS-7 cells) were plated at a density of 10^6 cells per 100-mm dish, and were transfected with 25 μg/dish plasmid DNA using electroporation at 230 V and 960 μFD with a gene pulser (Bio-Rad). Transfected cells were grown in 5 or 20% FCS-containing culture medium for at least 24–48 h. Various transfectants were analyzed for the expression of Tiam1 or HA-tagged (or GFP-tagged) Tiam1 mutant proteins by immunoblot, immunoprecipitation, and functional assays as described below.

Immunoprecipitation and Immunoblotting Techniques

SP-1 cells or COS cells (e.g., untransfected or transfected by various Tiam1 cDNAs including the full-length mouse Tiam1 cDNA [FL1591] or HA-tagged C1199 Tiam1 cDNA) were first extracted with a solution containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% NP-40 buffer, followed by solubilizing in SDS sample buffer, and analyzed by SDS-PAGE (with 7.5% gel). Separated polypeptides were transferred onto nitrocellulose filters. After blocking nonspecific sites with 3% BSA, the nitrocellulose filters were incubated with 5 μg/ml either of rabbit anti-Tiam1 or mouse anti-HA (or preimmune serum) plus peroxidase-conjugated goat anti–rabbit IgG or goat anti–mouse IgG (1:10,000 dilution), respectively. In controls, peroxidase-conjugated normal mouse IgG or preimmune rabbit IgG was also incubated with anti-Tiam1–mediated immunocomplex. The blots were developed using ECL chemiluminescence reagent (Amersham Life Science) according to the manufacturer's instructions.

In some cases, SP-1 cells (transfected with HA-tagged C1199 Tiam1 cDNA, or HA-tagged C1199 Tiam1Δ717-727 cDNA, or GFP-tagged Tiam1 fragment cDNA, or cotransfected with HA-tagged C1199 Tiam1 cDNA and GFP-tagged Tiam1 fragment cDNA) were immunoblotted with anti-HA antibody (5 μg/ml) or anti-GFP antibody (5 μg/ml), respectively, followed by incubation with HRP-conjugated goat anti–mouse IgG (1:10,000 dilution) at room temperature for 1 h.

SP-1 cells were also immunoprecipitated with rabbit anti-Tiam1 (5 μg/ml) or mouse antiankyrin antibodies (e.g., 5 μg/ml of either mouse anti-ANK3 antibody or mouse anti-ANK1 antibody), followed by immunoblotting/reblotting with ankyrin antibodies (e.g., 1 μg/ml mouse anti-ANK3 antibody, or 5 μg/ml mouse anti-ANK1 antibody, or 1 μg/ml rabbit anti-Tiam1), respectively, followed by incubation with HRP-conjugated goat anti–mouse IgG or goat anti–rabbit IgG (1:10,000 dilution) at room temperature for 1 h. In reblotting controls, both peroxidase-conjugated normal mouse IgG or rabbit preimmune IgG was also used. The blots were developed using ECL chemiluminescence reagent (Amersham Life Science) according to the manufacturer's instructions.

Effects of Synthetic Peptides on Ankyrin-Tiam1 Interaction

Nitrocellulose discs (1-cm diam) were coated with ~1 μg of a panel of synthetic peptides including the ankyrin-binding region peptide (^{717}GEGTDAVKRS^{727}L), a scrambled peptide (GRATLEGSDKV) and another Tiam1-related peptide (^{399}GTIKRAPFLG^{409}P; synthesized by Dr. Eric Smith, University of Miami). After coating, the unoccupied sites on the discs were blocked by incubation with a solution containing 20 mM Tris-HCl, pH 7.4, and 0.3% BSA at 4°C for 2 h. The discs were incubated with various concentration of ^{125}I-labeled cytoskeletal proteins (erythrocyte ankyrin/ARD/ankyrin's SBD/spectrin; ~3000 cpm/ng) at 4°C for 2 h in 1 ml binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% BSA).

In some experiments, ^{125}I-labeled Tiam1 (~3,000 cpm/ng) was incubated with ankyrin-coated beads in the presence of various concentrations (10^{-10}–10^{-6} M) of unlabeled synthetic peptide (e.g., ^{717}GEGTDAVKRS^{727}L or the scrambled sequence, GRATLEGSDKV, or another Tiam1-related peptide, ^{399}GTIKRAPFLG^{409}P) at 4°C for 2 h in 1 ml binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% BSA). ^{125}I-labeled Tiam1 fragment (~3,000 cpm/ng) was also incubated with beads containing 1.0 μg of each of the following four proteins: intact ankyrin, ARD, or spectrin binding domain of ankyrin (GFP-SBD), or GFP alone.

After binding, the peptide-coated discs (or cytoskeletal protein–conjugated beads) were washed three times in the binding buffer, and the radioactivity associated with the peptide-coated discs (or cytoskeletal protein–conjugated beads) was estimated. As a control, the ligands were also incubated with uncoated nitrocellulose discs (or beads) to determine the binding observed because of the stickiness of various ligands. Nonspecific binding was observed in these controls. In the peptide competition assay, the specific binding observed in the absence of any of the competing peptides is designated as 100%. The results represent an average of duplicate determinations for each concentration of the competing peptide used.

Binding of Ankyrin or ARD to Tiam1 In Vitro

Aliquots (0.5–1.0 μg of protein) of purified Tiam1 (e.g., intact Tiam1, or C1199 Tiam1, or Tiam1 fragment)–conjugated beads were incubated in 0.5 ml of binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% BSA, and 0.05% Triton X-100) containing various concentrations (10–800 ng/ml) of ^{125}I-labeled intact ankyrin (purified from human erythrocytes; 5,000 cpm/ng protein) or ^{125}I-labeled recombinant ARD fragment at 4°C for 4 h. Specifically, equilibrium binding conditions were determined by performing a time course (1–10 h) of ^{125}I-labeled ankyrin (or ARD) binding to Tiam1 at 4°C. The binding equilibrium was found to be established when the in vitro ankyrin (or ARD)-Tiam1 binding assay was conducted at 4°C after 4 h. After binding, beads were washed extensively in binding buffer, and the bead-bound radioactivity was counted.

As a control, ^{125}I-labeled ankyrin or ^{125}I-labeled ARD was also incubated with uncoated beads to determine the binding observed because of the nonspecific binding of various ligands. Nonspecific binding, which represented ~20% of the total binding, was always subtracted from the total binding. Our binding data are highly reproducible. The values expressed in the Results represent an average of triplicate determinations of three to five experiments with an SD less than ± 5%. In some cases, ^{125}I-ankyrin (1–10 ng) was incubated with a polyacrylamide gel containing purified Tiam1 (obtained from anti-Tiam1 affinity column chromatography) in the absence or the presence of 100-fold excess amount of unlabeled ankyrin/spectrin (in the same binding buffer as described above) for 1 h at room temperature. After incubation, the gel was washed five times with the same binding solution and analyzed by autoradiographic analyses.

An in vitro binding assay designed to measure the stoichiometry of GST-ARD fusion protein and C1199 Tiam1 was also carried out. Specifically, in each reaction, 15–60 μl of glutathione-Sepharose bead slurry containing GST-ARD or GST alone was suspended in 0.5 ml of binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% BSA, and 0.05% Triton X-100). Purified C1199 Tiam1 (0.5–1.0 μg) was added to the bead suspension in the absence or the presence of an excess amount of CBP-tagged Tiam1 fragment (100 μg) at 4°C for 4 h. After binding, the GST fusion protein was eluted with its associated C1199 Tiam1 using 150 μl of 50 mM Tris-HCl, pH 8.0, buffer containing 30 mM glutathione. The amount of eluted GST fusion protein and C1199 Tiam1 was determined by SDS-PAGE and Coomassie blue staining followed by densitometric scanning using a software NIH Image V1.54. The amount of ARD (mol) per C1199 Tiam1 (mol) was calculated. Values represent relative binding abilities averaged from three experiments ± SEM.

Binding of ^{125}I-Labeled Ankyrin to C1199 Tiam1 and the Mutant Protein

SP1 cells were transfected with HA-tagged C1199 Tiam1 cDNA, or HA-tagged C1199 Tiam1Δ717-727 cDNA, or vector alone. These transfectants were extracted with a solution containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% NP-40, and immunoprecipitated with anti-HA immunoaffinity beads. Subsequently, aliquots (50 ng proteins) of these beads were incubated with 0.5 ml of a binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% BSA, and 0.05% Triton X-100) in presence of various concentrations (10–400 ng/ml) of ^{125}I-labeled ankyrin (5,000 cpm/ng protein) at 4°C for 5 h. After binding, beads were washed extensively in binding buffer and the bead-bound radioactivity was counted.

As a control, ^{125}I-labeled ankyrin was also incubated with uncoated beads to determine the binding observed because of the nonspecific binding of the ligand. Nonspecific binding, which represented ~15–20% of the total binding, was always subtracted from the total binding. The values expressed in the Results represent an average of triplicate determinations of three to five experiments with an SD less than ± 5%.

Tiam1-mediated GDP/GTP Exchange for Rho GTPases

Purified *E. coli*–derived GST-tagged GTPases (e.g., Rac1, Cdc42, or RhoA; 20 pmol) were preloaded with GDP (30 μM) in 10 μl buffer containing 25 mM Tris-HCl, pH 8.0, 1 mM DTT, 4.7 mM EDTA, 0.16 mM $MgCl_2$, and 200 μg/ml BSA at 37°C for 7 min. To terminate preloading procedures, additional $MgCl_2$ was added to the solution (reaching a final concentration of 9.16 mM) as described previously (Zhang et al., 1995). Tiam1 was isolated from COS-7 cells (transfected with either the full-length Tiam1 cDNA or HA-tagged C1199 Tiam1 cDNA) or SP1 cells (transfected with various plasmid DNAs such as HA-tagged C1199 Tiam1 cDNA, GFP-tagged Tiam1 fragment cDNA, or HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA [as cotransfection] or vector alone) using anti-Tiam1 (or anti-HA or anti-GFP)–conjugated beads. In some cases, ankyrin-associated Tiam1 was isolated from SP1 cells (transfected with HA-tagged C1199 Tiam1 cDNA, GFP-tagged Tiam1 fragment cDNA, or HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA [as a cotransfection], or vector alone) using antiankyrin-conjugated beads.

Subsequently, 2 pmol of Tiam1, isolated from untransfected or transfected cells according to the procedures described above, was preincubated with no ankyrin or ankyrin (e.g., 1 μg/ml of either intact ankyrin or ARD), followed by adding to the reaction buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 100 μM AMP-PNP, 0.5 mg/ml BSA, and 2.5 μM GTP-γ-^{35}S (~1,250 Ci/mmol). Subsequently, 2.5 pmol GDP-loaded GST-tagged Rho GTPases (e.g., Rac1, RhoA, or Cdc42) or GDP-treated GST were mixed with the reaction buffer containing Tiam1 and GTP-γ-^{35}S to initiate the exchange reaction at room temperature. At various time points, the reaction of each sample was terminated by adding ice-cold termination buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 10 mM $MgCl_2$ as described previously (Michiels et al., 1995; Zhang et al., 1995). The termination reactions were filtered immediately through nitrocellulose filters, followed by one wash with the termination buffer. The filters were dissolved completely in scintillation fluid, and the radioactivity associated with the filters were measured by scintillation fluid. The amount of GTP-γ-^{35}S bound to Tiam1 or control sample (preimmune serum–conjugated Sepharose beads) in the absence of Rho GTPases (e.g., Rac1, Cdc42, or RhoA) was subtracted from the original values. Data represent an average of triplicates from three to five experiments. SD < 5%.

Double Immunofluorescence Staining

SP1 cells (untransfected or transfected with various plasmid DNAs such as HA-tagged C1199 Tiam1 cDNA, GFP-tagged Tiam1 fragment cDNA, or HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA [as a cotransfection], or vector alone) were first washed with PBS (0.1 M phosphate buffer, pH 7.5, and 150 mM NaCl) buffer and fixed by 2% paraformaldehyde. Subsequently, cells were rendered permeable by ethanol treatment followed by staining with different immunoreagents. Specifically, untransfected cells were incubated with rhodamine (Rh)-conjugated mouse anti-ANK3 (50 μg/ml) and fluorescein (FITC)-conjugated rabbit anti-Tiam1 (50 μg/ml), respectively. HA-tagged C1199 cDNA–transfected cells were stained with Rh-conjugated mouse anti-ANK3 antibody (50 μg/ml) and FITC-conjugated mouse anti-HA IgG (50 μg/ml), respectively. GFP-tagged Tiam1 fragment cDNA–transfected cells were labeled with Rh-conjugated anti-ANK3 (50 μg/ml). Some SP1 transfectants (cotransfected with Tiam1 fragment cDNA and HA-tagged C1199 Tiam1 cDNA) were stained with Rh-conjugated anti-HA (50 μg/ml) or Rh-conjugated anti-ANK3 (50 μg/ml), respectively. To detect nonspecific antibody binding, vector-transfected cells were labeled with Rh-conjugated anti-ANK3 (50 μg/ml) followed by incubating with FITC-conjugated anti-HA (50 μg/ml). No anti-HA labeling was observed in such control samples. In some experiments, GFP-tagged Tiam1 fragment cDNA–transfected cells were also incubated with Rh-labeled rabbit preimmune IgG (50 μg/ml). No nonspecific rhodamine staining was detected in these samples. The FITC- and Rh-labeled samples were examined with a confocal laser scanning microscope (MultiProbe 2001 inverted CLSM system; Molecular Dynamics).

Tumor Cell Migration and Invasion Assays

24 transwell units were used for monitoring in vitro cell migration and invasion as described previously (Merzak et al., 1994; Bourguignon et al., 1998b, 2000). Specifically, the 5-μm porosity polycarbonate filters coated with the reconstituted basement membrane substance Matrigel (Collabo-

rative Research) were used for the cell invasion assay (Merzak et al., 1994; Bourguignon et al., 1998b). The 5-μm porosity polycarbonate filters (without Matrigel coating) were used for the cell migration assay (Merzak et al., 1994; Bourguignon et al., 1998b, 2000). SP-1 cells transfected with various Tiam1-related cDNAs (e.g., full-length Tiam1 cDNA, HA-tagged C1199 Tiam1 cDNA, or GFP-tagged Tiam1 fragment cDNA, or HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA [cotransfection], or vector alone) ($\sim 10^{-4}$ cells/well in PBS, pH 7.2, untreated or treated with cytochalasin D [20 μg/ml] or DMSO alone) were placed in the upper chamber of the transwell unit. The growth medium containing high glucose DME supplemented by 10% FBS was placed in the lower chamber of the transwell unit. After an 18-h incubation at 37°C in a humidified 95% air/5% CO_2 atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell migration and invasion processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters by standard cell number counting methods as described previously (Merzak et al., 1994; Zhu and Bourguignon, 2000). Each assay was set up in triplicate and repeated at least five times. All data were analyzed statistically by *t* test and statistical significance was set at $P < 0.01$.

Results

Identification of the GEF, Tiam1 in Breast Tumor Cells (SP-1 Cells)

Rho GTPases such as Rac1 become activated when bound GDP is exchanged for GTP by a process catalyzed by GEFs such as Tiam1 (Habets et al., 1994). A Tiam1 transcript has been detected previously in breast cancer cells (Habets et al., 1995). In this study, we have analyzed Tiam1 expression (at the protein level) in SP-1 breast tumor cells. Immunoblot analysis, using anti-Tiam1 antibody designed to recognize the specific epitope located at the COOH terminus of Tiam1 molecule, reveals a single polypeptide (~200 kD; Fig. 1, lane 1). This 200-kD Tiam1-like molecule, expressed in SP-1 cells, is very similar to the Tiam1 detected in COS-7 cells that were transiently transfected with the full-length Tiam1 cDNA (Fig. 1, lane 2) or NH_2 terminally truncated C1199 Tiam1 cDNA (Fig. 1, lane 3 revealing primarily C1199 Tiam1 [160 kD] and a low level of endogenous Tiam1 [200 kD]). We believe that the Tiam1 detected in SP-1 cells or COS-7 transfectants, revealed by anti-Tiam1–mediated immunoblot, is specific since no protein is detected in these cells using preimmune rabbit IgG (Fig. 1, lanes 4–6).

To confirm that the Tiam1-like molecule functions as a GDP/GTP exchange factor (or a GDP-dissociation stimulator protein) for Rac1, we have isolated Tiam1 from SP-1 cells using anti-Tiam1–conjugated Sepharose beads. Our results indicate that SP1's Tiam1 activates GDP/GTP exchange on GST-Rac1 (Fig. 2 A, a) and, to a lesser extent, on GST-Cdc42 (Fig. 2 A, b) and GST-RhoA (Fig. 2 A, c). The initial onset of the exchange reaction on GST-Rac1

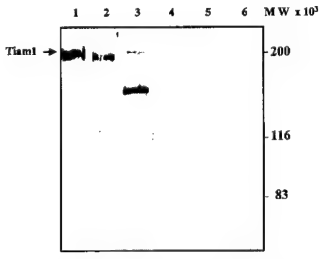

Figure 1. Detection of Tiam1 expression in SP-1 cells or COS-7 transfectants. SP-1 and COS-7 cells, which were transfected with the full-length Tiam1 cDNA (FL1591) or NH_2 terminally truncated C1199 Tiam1 cDNA or vector alone, were solubilized in SDS sample buffer and analyzed by SDS-PAGE and immunoblot as described in Materials and Methods. (lane 1) Anti-Tiam1–mediated immunoblot of SP-1 cells. (lane 2) Anti-Tiam1–mediated immunoblot of COS-7 cells transfected with the full-length Tiam1 cDNA (FL1591). (lane 3) Anti-Tiam1–mediated immunoblot of COS-7 cells transfected with the NH_2 terminally truncated C1199 Tiam1 cDNA. (lane 4) Immunoblot of SP-1 cells with preimmune rabbit serum. (lane 5) Immunoblot of COS-7 cells, which were transfected with Tiam1 cDNA [FL1591], with preimmune rabbit serum. (lane 6) Immunoblot of COS-7 cells, which were transfected with the NH_2 terminally truncated C1199 Tiam1 cDNA, with preimmune rabbit serum.

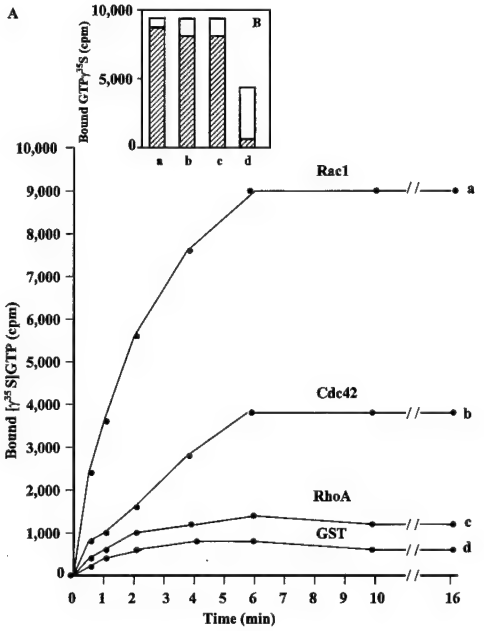

Figure 2. Tiam1-mediated GDP/GTP exchange for Rho GTPases. Purified *E. coli*-derived GST-tagged GTPases (e.g., Rac1, Cdc42, or RhoA) were preloaded with GDP. First, 2 pmol Tiam1 that was isolated from SP1 cells or COS-7 transfectants was added to the reaction buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 100 μM AMP-PNP, 0.5 mg/ml BSA, and 2.5 μM GTP-γ-^{35}S (~1,250 Ci/mmol). Subsequently, 2.5 pmol GDP-loaded GST-tagged Rho GTPases (e.g., Rac1, RhoA, Cdc42, or GST alone) were mixed with the reaction buffer containing Tiam1 and GTP-γ-^{35}S to initiate the exchange reaction at room temperature. At various time points, the reaction of each sample was terminated by adding ice-cold termination buffer as described in Materials and Methods. The termination reactions were filtered immediately through nitrocellulose filters, and the radioactivity associated with the filters were measured by scintillation fluid. The amount of GTP-γ-^{35}S bound to Tiam1 or control sample (preimmune serum–conjugated Sepharose beads) in the absence of Rho GTPases (e.g., Rac1, Cdc42, or RhoA) was subtracted from the original values. Data represent an average of triplicates from three to five experiments. SD < 5%. (A) Kinetics of GTP-γ-^{35}S bound to GDP-loaded GST-Rac1 (a), GST-Cdc42 (b), or GST-RhoA (c), or GST alone (d) in the presence of Tiam1 (isolated from SP-1 cells). (B) The maximal level of GTP-γ-^{35}S bound to GST-Rac1 in the presence of Tiam1 isolated from SP1 grown in 5% FCS (a, shaded bar) or 20% FCS (a, blank bar); or the full-length Tiam1 (1,591) isolated from COS-7 transfectants grown in 5% FCS (b, shaded bar) or 20% FCS (b, blank bar); or the C1199 Tiam1 isolated from COS-7 transfectants grown in 5% FCS (c, shaded bar), or 20% FCS (c, blank bar); or Tiam1 isolated from vector-transfected COS-7 cells grown in 5% FCS (d, shaded bar) or 20% FCS (d, blank bar).

occurs within 0.5–1 min after the addition of Tiam1, and the reaction reaches its maximal level ~16 min after Tiam1 addition (Fig. 2 A, a). In contrast, the initial rate of Tiam1-catalyzed GDP/GTP exchange on Cdc42 (Fig. 2 A, b) and RhoA (Fig. 2 A, c) appears to be significantly lower than that detected on Rac1 (Fig. 2 A, a). In the control samples, the amount of [^{35}S]GTP-γ–S associated with GST alone is found to be significantly decreased (Fig. 2 A, d). Further analysis indicates that the ability of Tiam1 isolated from SP-1 cells to promote GDP/GTP exchange on Rac1 (Fig. 2 B, a) is identical to that carried out by the Tiam1 isolated from COS-7 transfected with the full-length Tiam1 cDNA (Fig. 2 B, b) or NH_2 terminally truncated C1199 Tiam1 cDNA (Fig. 2 B, c). Therefore, we believe that the Tiam1 in SP-1 cells clearly functions as a GDP/GTP exchange factor for Rho-like GTPases such as Rac1 GTPase.

We have also noticed that Tiam1 isolated from nontransfected COS-7 cells grown in the presence of 20% FCS is capable of catalyzing GDP/GTP exchange on Rac1 at a much higher level (Fig. 2 B, d, blank bar) than Tiam1 isolated from nontransfected COS-7 cells grown in the presence of 5% FCS (Fig. 2 B, d, shaded bar). This observation is consistent with the previous findings that some serum components play an important role in upregulating the ability of Tiam1 to promote GDP/GTP exchange on Rac1 (Stam et al., 1998). In SP1 cells (Fig. 2 B, a, blank and shaded bars) or Tiam1 cDNA–transfected COS-7 cells (Fig. 2 B, b and c, blank and shaded bars), neither high nor low serum causes significant changes in the ability of Tiam1 to catalyze GDP/GTP exchange on Rac1. These differential serum effects on the activity of Tiam1 isolated from low or high Tiam1-expressing cells await future investigation.

Interaction between Tiam1 and the Cytoskeletal Proteins, Ankyrin

Certain cytoskeleton proteins, such as ankyrin, are known to be involved in regulating a variety of cellular activities (Bennett, 1992; Bennett and Gilligan, 1993; Bourguignon, 1996; Bourguignon et al., 1998a; De Matteis and Morrow, 1998). Both ankyrin1 (ANK1) and ankyrin 3 (ANK3) have been shown to be expressed in breast tumor cells (Bourguignon et al., 1998b, 1999). In this study, we have carried out anti-ANK1 or anti-ANK3–mediated immunoprecipitation of SP-1 cellular proteins, followed by anti-Tiam1 immunoblot (Fig. 3, A and B, lane 2) and anti-ANK1 (Fig. 3 A, lane 3)/ANK3 (Fig. 3 B, lane 3) immunoblot, respectively. Our results indicate that the Tiam1 band is revealed in antiankyrin (e.g., ANK1 or ANK3)–mediated immunoprecipitated materials (Fig. 3, A and B, lane 2). Apparently, Tiam1 is coprecipitated with ANK1 and/or ANK3 (revealed by reblotting with anti-ANK1/ANK3 antibody; Fig. 3, A and B). In control samples, immunoblotting of anti-ANK1 or anti-ANK3–immunoprecipitated material using rabbit preimmune serum (Fig. 3, A and B, lane 1) does not reveal any protein associated with this material. Anti-Tiam1–mediated immunoprecipitation of SP-1 cellular proteins, followed by anti-ANK1 (Fig. 3 A, lane 4) or anti-ANK3 (Fig. 3 B, lane 4)–mediated immunoblot also shows that both ANK1 (Fig. 3 A, lane 4) and ANK3 (Fig. 3 B, lane 4) can be coprecipitated with Tiam1 (revealed by reblotting with anti-Tiam1 antibody; Fig. 3, A and B, lane 5). In controls, very little material is detected in this anti-Tiam1–mediated immunocomplex using either normal mouse IgG (Fig. 3, A and B, lane 6) or rabbit preimmune serum–mediated immunoblot (Fig. 3 A, lane 7). These findings clearly establish the fact that Tiam1 and ankyrin (e.g., ANK1 and ANK3) are closely associated with each other as an in vivo complex in breast tumor cells.

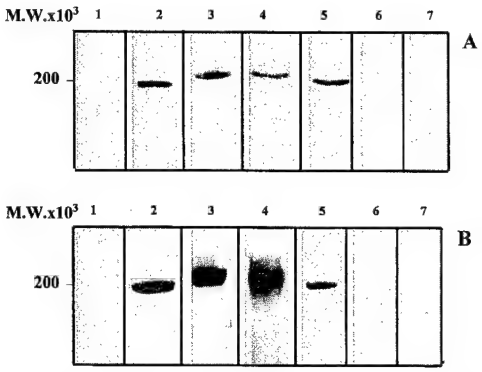

Figure 3. Detection of Tiam1–ankyrin complex in SP1 cells. SP1 cells (5×10^5 cells) were solubilized by 1% NP-40 buffer and processed for antiankyrin or anti-Tiam1–mediated immunoprecipitation, followed by immunoblotting with anti-Tiam1 or anti-ANK1 (or anti-ANK3) antibody, respectively, as described in Materials and Methods. (A) Analysis of Tiam1–ANK1 complex (lane 1). Anti-ANK1–mediated immunoprecipitation followed by immunoblotting with rabbit preimmune serum. (lanes 2 and 3) Detection of Tiam1 in the complex by mouse anti-ANK1–mediated immunoprecipitation, followed by immunoblotting with rabbit anti-Tiam1 antibody (lane 2) or reblotting with mouse anti-ANK-1 antibody (lane 3). (lanes 4–7) Detection of ANK1 in the complex by rabbit anti-Tiam1–mediated immunoprecipitation, followed by immunoblotting with mouse anti-ANK1 antibody (lane 4) or reblotting with rabbit anti-Tiam1 antibody (lane 5), or peroxidase-conjugated normal mouse IgG (lane 6) or peroxidase-conjugated rabbit preimmune IgG (lane 7). (B) Analysis of Tiam1-ANK3 complex: (lane 1) anti-ANK3-mediated immunoprecipitation followed by immunoblotting with rabbit preimmune serum. (lanes 2 and 3) Detection of Tiam1 in the complex by mouse anti-ANK3-mediated immunoprecipitation, followed by immunoblotting with rabbit anti-Tiam1 antibody (lane 2) or reblotting with mouse anti-ANK-3 antibody (lane 3). (lanes 4–7) Detection of ANK3 in the complex by rabbit anti-Tiam1–mediated immunoprecipitation, followed by immunoblotting with mouse anti-ANK3 antibody (lane 4) or reblotting with rabbit anti-Tiam1 antibody (lane 5), or peroxidase-conjugated normal mouse IgG (lane 6), or peroxidase-conjugated rabbit preimmune IgG (lane 7).

Further analyses using an in vitro binding assay show that ^{125}I-labeled ankyrin (i.e., erythrocyte ankyrin [ANK1]) binds Tiam1, which was isolated from SP1 cells, specifically (Fig. 4 A, a). In addition, we have used ^{125}I-labeled ankyrin to bind purified Tiam1 (isolated from SP-1 cells) on a gel (Fig. 4 B, a). Our data indicate that Tiam1 binds to ankyrin (ANK1; Fig. 4 B, a) directly. In the presence of an excess amount of unlabeled ankyrin, the binding between ankyrin and Tiam1 is greatly reduced (Fig. 4, A and B, b). Other cytoskeletal proteins, such as spectrin,

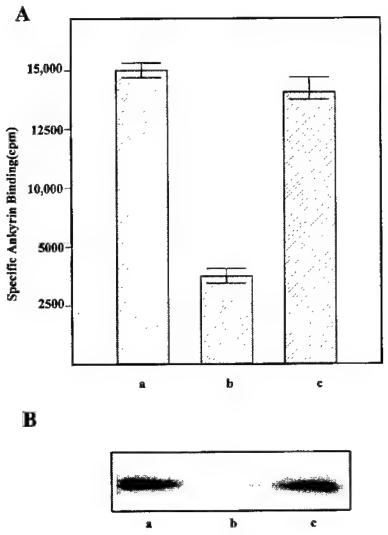

Figure 4. Binding interaction between Tiam1 and the cytoskeletal protein ankyrin. (A) Tiam1 (isolated from SP-1 cells) bound to the anti-Tiam1 immunobeads were incubated with ^{125}I-labeled ankyrin (5,000 cpm/ng protein) in the absence (a) or the presence of 100-fold excess of unlabeled ankyrin (b) or spectrin (c). After binding, the immunobeads were washed extensively in binding buffer and the bead-bound radioactivity was estimated. (B) Autoradiogram of ^{125}I-labeled ankyrin binding to a polyacrylamide gel containing purified Tiam1 (isolated from SP-1 cells) in the absence (a) or the presence of 100-fold excess of unlabeled ankyrin (b) or spectrin (c).

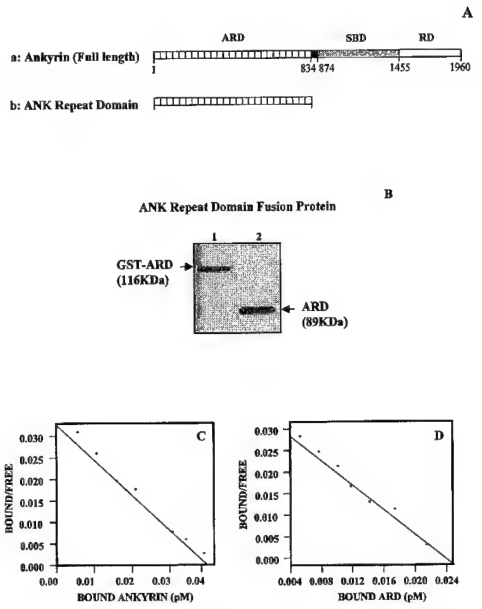

Figure 5. Ankyrin structure and ankyrin repeat domain (ARD) fusion protein. (A, a) Schematic illustration of functional domains in full-length ankyrin: ankyrin repeat domain (ARD), spectrin binding domain (SBD), and regulatory domain (RD). (A, b) ARD cDNA was constructed according to the strategy described in Materials and Methods. This ARD cDNA construct encodes for the NH_2-terminal region of the ankyrin membrane binding domain with a tandem array of 24 ankyrin repeats. (B) A Coomassie blue stain of the 116-kD GST-ARD fusion protein purified by affinity column chromatography (lane 1), and the 89-kD ARD (lane 2) after the removal of GST by thrombin digestion. (C and D) Scatchard plot analyses of the equilibrium binding between ^{125}I-labeled ankyrin and Tiam1. Various concentrations of ^{125}I-labeled ankyrin (e.g., intact erythrocyte ankyrin [ANK1] or ARD) were incubated with purified Tiam1-coupled beads at 4°C for 4 h. After binding, beads were washed extensively in binding buffer and the bead-bound radioactivity was counted. As a control, ^{125}I-labeled ankyrin or ^{125}I-labeled ARD was also incubated with uncoated beads to determine the binding observed because of the nonspecific binding of various ligands. Nonspecific binding, which represented ~20% of the total binding, was always subtracted from the total binding. Our binding data are highly reproducible. Scatchard plot analysis of the equilibrium binding data between ^{125}I-labeled intact erythrocyte ankyrin (ANK1) and Tiam1 (C); and Scatchard plot analysis of the equilibrium binding data between ^{125}I-labeled ARD and Tiam1 (D).

do not interfere with ankyrin binding to Tiam1 (Fig. 4, A and B, c). However, the precise functional domain of ankyrin involved in Tiam1 binding remains to be determined.

The NH_2-terminal region of ankyrin's membrane binding domain (Fig. 5 A, a) is comprised of a tandem array of 24 ankyrin repeats (so-called ankyrin repeat domain, ARD; Fig. 5 A, b). The question of whether the membrane-binding domain of ankyrin (in particular, ARD) is involved in Tiam1 binding is now addressed in this study. First, the pGEX-2TK recombinant plasmid encoding ARD (NH_2-terminal portion of ankyrin, from amino acids 1 to 834) was constructed with a GST tag and expressed in *E. coli* (Zhu and Bourguignon, 2000). The purified GST-tagged ARD fusion protein is expressed as a 116-kD protein (Fig. 5 B, lane 1). After the removal of GST tag by thrombin digestion, the ARD itself is found to be an 89-kD polypeptide (Fig. 5 B, lane 2), which is similar to the 89-kD ARD obtained by enzymatic digestion of erythrocyte ankyrin (Davis and Bennett, 1990).

Next, we have used the ARD fragment of ANK3 (GST-ARD) and purified Tiam1 to identify the exact Tiam1 binding site(s) on the ankyrin molecule. Specifically, we have tested the binding of Tiam1 to ^{125}I-labeled intact erythrocyte ankyrin (ANK1), or ^{125}I-labeled GST-ARD fragment of ANK3, under equilibrium binding conditions. Scatchard plot analyses indicate that intact erythrocyte ankyrin (ANK1) binds to Tiam1 at a single site (Fig. 5 C) with high affinity (an apparent dissociation constant [K_d] of ~0.72 nM). This ankyrin–Tiam1 binding interaction is comparable in affinity to Tiam1 binding (K_d ~1.42 nM) to ANK3's ARD fragment (Fig. 5 D). These findings strongly support the notion that ankyrin (in particular, the ARD) is involved in the Tiam1 binding site.

Determination of Tiam1's Ankyrin-binding Domain

Previous studies indicate that Tiam1's NH_2-terminal pleckstrin homology (PHn) domain and an adjacent protein interaction domain (i.e., a sequence between amino acids 393 and 738 of Tiam1; Fig. 6 A, a–c) is required for the activation of Rac1 signaling pathways leading to membrane ruffling and c-Jun NH_2-terminal kinase activation (Michiels

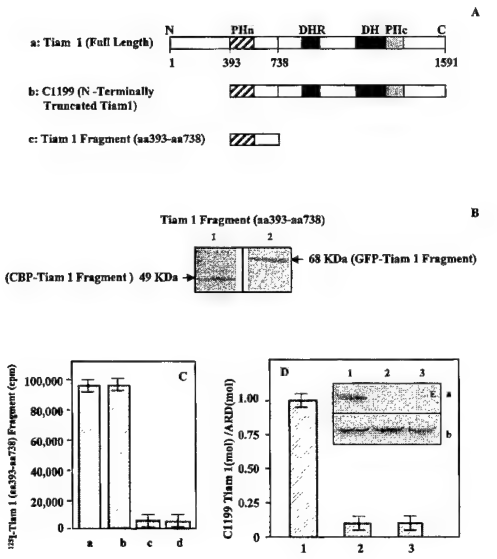

Figure 6. Properties of Tiam1 and Tiam1 mutant proteins. (A, a) The full-length Tiam1 contains DH, *dbl* homology domain; DHR, *discs-large* homology domain; two pleckstrin homology (PH) domains (including the NH_2-terminal PH [PHn] and the COOH-terminal PH [PHc]). (A, b) The NH_2 terminally truncated C1199 Tiam1 encodes the COOH-terminal 1,199 amino acids. (A, c) The Tiam1 fragment encodes the sequence between amino acids 393 and 738. (B) Characterization of Tiam1 fragment (amino acids 393–738) fusion proteins. Coomassie blue staining of *E. coli*–derived CBP-Tiam1 fragment fusion protein purified by calmodulin affinity column chromatography (lane 1); and GFP-tagged Tiam1 fragment fusion purified by anti-GFP–conjugated affinity column chromatography (lane 2). (C, a) Binding of ^{125}I-Tiam1 fragment to ankyrin. (C, b) Binding of ^{125}I-Tiam1 fragment to ARD. (C, c) Binding of ^{125}I-Tiam1 fragment to the spectrin binding domain of ankyrin. (C, d) Binding of ^{125}I-Tiam1 fragment to spectrin. (D and E) Binding analysis between GST-ARD and the recombinant C1199 Tiam1 in vitro. In each reaction, glutathione-Sepharose bead slurry containing GST-ARD or GST alone was suspended in the binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% BSA, and 0.05% Triton X-100). Purified C1199 Tiam1 (0.5–1.0 μg) was added to the bead suspension in the absence or the presence of an excess amount of CBP-tagged Tiam1 fragment (100 μg) at 4°C for 4 h. After binding, the GST fusion protein was eluted with its associated C1199 Tiam1 using 150 μl of 50 mM Tris-HCl, pH 8.0, buffer containing glutathione. The amount of eluted GST fusion protein and C1199 Tiam1 was determined by SDS-PAGE and Coomassie blue staining, followed by densitometric scanning using a software NIH Image V1.54. The amount of ARD (mol) per C1199 Tiam1 (mol) was calculated. Values represent relative binding abilities averaged from three experiments ± SEM. (D) The amount of C1199 Tiam1 (mol) associated with GST-ARD (mol) was measured in the absence (lane 1) or the presence of the recombinant Tiam1 fragment (lane 2) or C1199 Tiam1 associated with GST-coated beads (lane 3) using SDS-PAGE and Coomassie blue staining followed by densitometric analyses. (E) Coomassie blue staining of C1199 Tiam1 associated with GST-ARD in the absence (lane 1) or the presence of recombinant Tiam1 fragment (lane 2) or C1199 Tiam1 associated with GST-coated beads (lane 3).

et al., 1997; Stam et al., 1997). Using a 49-kD *E. coli*–derived CBP-tagged Tiam1 fragment (i.e., amino acids 393–738 of Tiam1; Fig. 6 B, lane 1) and an in vitro binding assay (Fig. 6 C), we have detected a specific binding interaction between the Tiam1 fragment and ankyrin (Fig. 6 C, a) and ARD (Fig. 6 C, b) but not the spectrin binding domain of ankyrin (Fig. 6 C, c) or spectrin (Fig. 6 C, d).

Furthermore, we have evaluated the binding interaction between GST-ARD fusion protein and the recombinant C1199 Tiam1 (NH_2 terminally truncated Tiam1; Fig. 6 D). First, glutathione-Sepharose beads containing GST-ARD were incubated with C1199 Tiam1 in the absence (Fig. 6, D and E, lane 1) or the presence of an excess amount of Tiam1 fragment (Fig. 6, D and E, lane 2). In controls, C1199 Tiam1 was also added to Sepharose beads containing GST alone (Fig. 6, D and E, lane 3). After binding, the GST fusion protein was eluted with its associated C1199 Tiam1 using a buffer containing glutathione. The amount of eluted GST fusion protein and C1199 Tiam1 was determined by SDS-PAGE and Coomassie blue staining (Fig. 6 E) followed by densitometric scanning analyses (Fig. 6 D). Our results indicate that the stoichiometry of ARD–C1199 Tiam1 interaction is ~1:1 (Fig. 6 D, lane 1, and Fig. 6 E, lane 1, a and b). In the presence of an excess amount (~100-fold) of recombinant Tiam1 fragment, the binding between ankyrin ARD and C1199 Tiam1 is significantly reduced (Fig. 6, D and E, lane 2, a and b). The control beads containing GST alone fail to bind C1199 Tiam1 (Fig. 6 D, lane 3, and Fig. 6 E, lane 3, a and b). These observations suggest that ankyrin ARD directly interacts with Tiam1, and that the ankyrin-binding domain (ARD)–containing Tiam1 fragment act as a potent competitive inhibitor of Tiam1 binding to ankyrin in vitro.

Protein sequence analyses show that Tiam1 contains the sequence ^{717}GEGTDAVKRS^{727}L (in mouse), or ^{717}GEGTEAVKRS^{727}L (in human) that shares a great deal of sequence homology with the ankyrin-binding domain of the cell adhesion receptor, CD44 family (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998). To test whether the sequence GEGTDAVKRSL of Tiam 1 protein is in fact involved in ankyrin binding, we have examined the ability of an 11–amino acid synthetic peptide, identical to GEGTDAVKRSL, to bind various cytoskeletal proteins. As shown in Table I, this synthetic peptide binds specifically to intact ankyrin and the ARD, but not the SBD of ankyrin or other cytoskeletal proteins such as spectrin. Control peptides, containing the scrambled sequence (GRATLEGSDKV) with the same amino acid composition as that of the synthetic peptide or another peptide (GTIKRAPFLGP) from a different region (i.e., the sequence between amino acids 399 and 409) of Tiam1, fail to bind any cytoskeletal proteins tested (Table I).

We have also used the synthetic peptide corresponding to Tiam1's amino acid 717–727 sequence to compete for the binding of purified Tiam1 to ankyrin. As shown in Fig. 7 A (c), the synthetic peptide competes effectively with Tiam1 to bind ankyrin with an apparent inhibition constant (K_i) ~0.5 nM. However, control peptides such as GRATLEGSDKV (Fig. 7 A, a) or GTIKRAPFLGP (Fig. 7 A, b) do not compete at all with Tiam1 in ankyrin binding. These results suggest that the amino acid 717–727 sequence of Tiam1 is a critical part of the ankyrin-binding domain of Tiam1. Finally, we have constructed an HA-tagged C1199 Tiam1 deletion mutant lacking the ankyrin binding sequence, amino acids 717–727 (designated as C1199 Tiam1Δ717-727; Fig. 7 B, b). The truncated C1199

Table I. Binding of ^{125}I-Labeled Cytoskeletal Proteins to Synthetic Peptides

	nM × CPM Bound
Binding to GEGTDAVKRSL (the sequence between amino acids 717 and 727 of Tiam1)	
^{125}I-Labeled ankyrin	15,260 ± 120
^{125}I-Labeled ARD	14,560 ± 105
^{125}I-Labeled ankyrin's spectrin binding domain	770 ± 22
^{125}I-Labeled spectrin	850 ± 34
Binding to GRATLEGSDKV (the scrambled sequence)	
^{125}I-Labeled ankyrin	1,020 ± 36
^{125}I-Labeled ARD	920 ± 29
^{125}I-Labeled ankyrin's spectrin binding domain	901 ± 24
^{125}I-Labeled spectrin	996 ± 27
Binding to GTIKRAPFLGP (the sequence between amino acids 399 and 409 of Tiam1)	
^{125}I-Labeled ankyrin	899 ± 23
^{125}I-Labeled ARD	854 ± 17
^{125}I-Labeled ankyrin's spectrin binding domain	842 ± 19
^{125}I-Labeled spectrin	863 ± 20

^{125}I-labeled cytoskeletal proteins (e.g., intact ankyrin [100 ng] or ARD [100 ng] or spectrin binding domain of ankyrin [100 ng] or spectrin [100 ng] were incubated with nitrocellulose discs coated with either the synthetic peptide GEGTDAVKRSL (corresponding to the sequence between amino acids 717 and 727 of Tiam1), or the scrambled peptide GRATLEGSDKV, or another Tiam1-related peptide, GTIKRAPFLGP (corresponding to the sequence between amino acids 399 and 409 of Tiam1) at 4°C for 4 h as described in Materials and Methods. As a control, the radiolabeled ligands including ^{125}I-labeled ankyrin, ^{125}I-labeled ARD, and ^{125}I-labeled spectrin were also incubated with uncoated beads to determine the binding observed because of the nonspecific binding of various ligands. Nonspecific binding, which represented ~20% of the total binding, was always subtracted from the total binding.

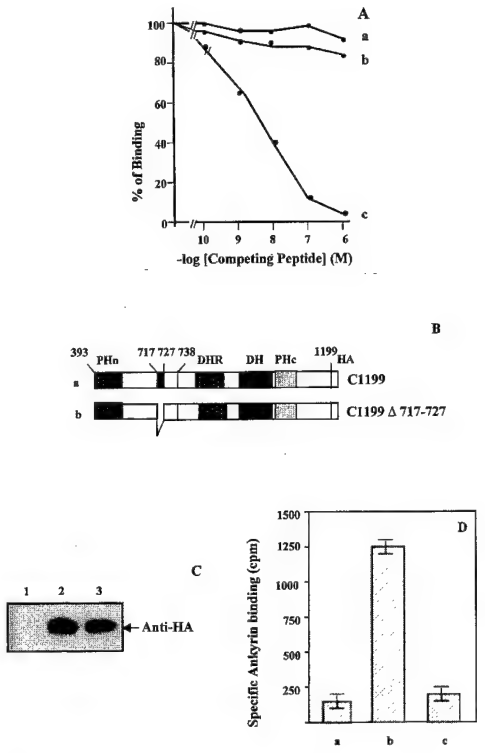

Figure 7. Identification of the ankyrin binding domain of Tiam1. (A) ^{125}I-labeled Tiam1 was incubated with ankyrin-coated beads in the presence of various concentrations of unlabeled synthetic peptide (GEGTDAVKRSL, corresponding to the sequence between amino acids 17 and 727 of Tiam1) (c), or the scrambled sequence (GRATLEGSDKV; a), or another Tiam1-related peptide (GTIKRAPFLGP, corresponding to the sequence between amino acids 399 and 409 of Tiam1; b) as described in Materials and Methods. The specific binding observed in the absence of any of the competing peptides is designated as 100%. The results represent an average of duplicate determinations for each concentration of the competing peptide used. (B) Schematic illustration of the in vitro mutagenesis approach used in this study. Both C1199 Tiam1 (a) and C1199 Tiam1 717-727 (lacking the sequence between amino acids 17 and 727; b) were constructed according to the strategy described in Materials and Methods. (C) Anti-HA–mediated immunoblot of SP-1 cells transiently transfected with vector alone (lane 1), or HA-tagged C1199 Tiam1 cDNA (lane 2), or HA-tagged C1199 Tiam1 717-727 cDNA (lane 3). (D) The amount of ^{125}I-ankyrin binding to anti-HA–mediated immunoprecipitates isolated from SP-1 cells transfected with vector alone (a), or HA-tagged C1199 Tiam1 cDNA (b), or HA-tagged C1199 Tiam1 717-727 cDNA (c).

Tiam1 717-727 cDNA (Fig. 7 B, b) and the wild-type C1199 Tiam1 (Fig. 7 B, a) were transiently transfected into SP-1 cells. Our results indicate that both the C1199 Tiam1Δ717-727 mutant (Fig. 7 C, lane 3) and the wild-type C1199 Tiam1 (Fig. 7 C, lane 2) are expressed as a 160-kD polypeptide in SP-1 transfectants using anti-HA–mediated immunoblotting. No protein band was detected in vector-transfected SP-1 cells (Fig. 7 C, lane 1). In vitro binding data reveal that there is a strong binding interaction between ankyrin and HA-tagged C1199 (Fig. 7 D, b). In contrast, the HA-tagged C1199 Tiam1Δ717-727 mutant protein isolated from SP-1 transfectants displays a drastic reduction (~90–95% inhibition) in ankyrin-binding ability (Fig. 7 D, c) compared with the HA-tagged wild-type C1199 Tiam1 (Fig. 7 D, b). No ankyrin binding is observed in materials associated with anti-HA beads using cell lysate isolated from vector-transfected cells (Fig. 7 D, a). These findings suggest that the amino acid 717–727 region is critical for the interaction of Tiam1 with ankyrin.

Most importantly, we have found that the binding of ankyrin (e.g., erythrocyte ankyrin [ANK1], Fig. 8 A, or ANK3's ARD, Fig. 8 B) to Tiam1 significantly increases the GDP/GTP exchange activity of Rac1 GTPase as compared with untreated Tiam1-mediated Rac1 activation (Fig. 8 C). The SBD of ankyrin or other cytoskeletal proteins, such as spectrin, fails to stimulate Tiam1-mediated GDP/GTP exchange on Rac1 GTPase (data not shown). Therefore, we believe that ankyrin binding to Tiam 1 plays a pivotal role in the upregulation of Tiam 1-mediated GDP/GTP exchange activity of Rho-like GTPases (e.g., Rac1).

Effect of Tiam1 or the Tiam1 Fragment on Rac1 Activation, Tumor Cell Invasion, and Migration

Previous studies have indicated that both ankyrin and Tiam1 are closely associated with certain tumor-specific behaviors, characterized by an invadopodia structure (or membranous projections) during epithelial tumor cell mi-

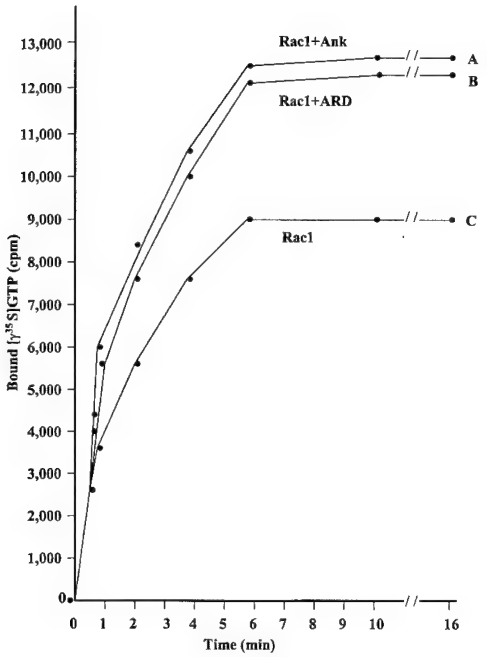

Figure 8. Stimulation of Tiam1-catalyzed GDP/GTP exchange activity by ankyrin. Purified *E. coli*–derived GST-tagged GTPases (e.g., Rac1, Cdc42, or RhoA) was preloaded with GDP. Subsequently, 2 pmol of Tiam1 (isolated from untransfected or transfected cells according to the procedures described above) was preincubated with no ankyrin or ankyrin (e.g., intact ankyrin or ARD; 1 μg/ml), followed by adding to the reaction buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 100 μM AMP-PNP, 0.5 mg/ml BSA, and 2.5 μM GTP-γ-^{35}S (~1,250 Ci/mmol). Subsequently, 2.5 pmol of GDP-loaded GST-tagged Rho GTPases (e.g., Rac1, Rac1, or Cdc42) was mixed with the reaction buffer containing Tiam1 and GTP-γ-^{35}S to initiate the exchange reaction at room temperature. At various time points, the reaction of each sample was terminated by adding ice-cold termination buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 10 mM $MgCl_2$ as described in Materials and Methods. The termination reactions were filtered immediately through nitrocellulose filters, and the radioactivity associated with the filters was measured by scintillation fluid. The amount of GTP-γ-^{35}S bound to Tiam1 or control sample (preimmune serum–conjugated Sepharose beads) in the absence of Rho GTPases (e.g., Rac1, Cdc42, or RhoA) was subtracted from the original values. Data represent an average of triplicates from three to five experiments. SD < 5%. (A–C) Kinetics of GTP-γ-^{35}S bound to GDP-loaded GST-Rac1 by Tiam1 (isolated from SP-1 cells) in the absence (C) or in the presence of ankyrin, e.g., intact erythrocyte ankyrin (ANK1; A) or ARD fragment (B).

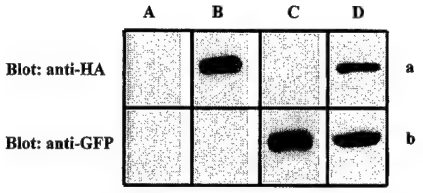

Figure 9. Transfection of SP1 cells with HA-tagged C1199 Tiam1 cDNA (A) or GFP-tagged Tiam1 fragment cDNA (B) or cotransfection of HA-tagged C1199 Tiam1 cDNA and GFP-tagged Tiam1 fragment cDNA (C). Detection of C1199 Tiam1 expression by anti-HA–mediated immunoblot in HA-tagged C1199 Tiam1 cDNA–transfected cells (B, a) or in vector-transfected cells (A, a). Detection of Tiam1 fragment expression by anti-GFP–mediated immunoblot in GFP-tagged Tiam1 fragment cDNA–transfected cells (C, b) or vector-transfected cells (A, b). Detection of coexpression of C1199 Tiam1 and Tiam1 fragment by immunoblotting of cells (cotransfected with HA-tagged C1199 Tiam1 cDNA and GFP-tagged Tiam1 fragment cDNA) with anti-HA antibody (D, a) and anti-GFP antibody (D, b), respectively. In controls, no signal was detected in HA-tagged C1199 Tiam1 cDNA–transfected cells or GFP-tagged Tiam1 fragment cDNA–transfected cells using anti-GFP (B, b) or anti-HA (C, a)–mediated immunoblotting, respectively.

gration (Bourguignon et al., 1998a,b, 2000; Zhu and Bourguignon, 2000). In this study, using double immunolabeling staining, we have observed that both ankyrin (Fig. 10 A) and Tiam1 (Fig. 10 B) are colocalized in the plasma membrane and long projections of SP1 cells (Fig. 10 C). Furthermore, we have transiently transfected breast tumor cells (e.g., SP-1 cells) with HA-tagged NH_2 terminally truncated C1199 Tiam1 cDNA. Our results show that the C1199 Tiam1 is expressed as a 160-kD protein (Fig. 9 B, a) detected by anti-HA–mediated immunoblot in SP1 cells. No protein band was detected in vector-transfected SP1 cells by anti-HA–mediated immunoblotting (Fig. 9 A, a).

Double immunofluorescence staining data show that ankyrin (Fig. 10 D) and C1199 Tiam1 (Fig. 10 E) are also colocalized on the plasma membrane–related long projections of these C1199 Tiam1 cDNA–transfected cells (Fig. 10 F). Furthermore, we have demonstrated that transfection of SP1 cells with C1199 Tiam1 cDNA stimulates ankyrin-associated Tiam1-catalyzed GDP/GTP exchange on Rac1 (Fig. 11 a), and induces a significant amount of increase in breast tumor cell invasion (Table II, A) and migration (Table II, B) as compared with vector-transfected SP1 transfectants (Fig. 11 b and Table II, A and B). These results are consistent with previous findings indicating that transfection of NIH3T3 cells with the NH_2 terminally truncated C1199 Tiam1 cDNA confers potent oncogenic properties (Van Leeuwen et al., 1995).

Treatment of SP1 cells (e.g., untransfected or transfected cells) with certain agents (e.g., cytochalasin D, a microfilament inhibitor) causes a remarkable inhibition of tumor cell invasion (Table II A) and migration (Table II B). Tiam1-Rac1 signaling initiates oncogenic cascades including c-Jun kinase (JNK) activation, which triggers gene transcription through c-jun and promotes cell transformation (Michiels et al., 1995, 1997). In addition, Tiam1-activated Rac1 stimulates the novel family of serine/threonine kinases, p-21 activated kinases (Manser et al., 1994; Knaus et al., 1995; Bagrodia and Cerione, 1999), which mediates actin assembly and induce the formation of membrane ruffling and lamellipodia (membrane projections). In fact, cytoskeleton-associated membrane projections are often tightly linked to matrix degrading enzymes during breast tumor cell invasion and migration (Bourguignon et al., 1998b). These findings suggest that Tiam1-Rac1 signaling and selective effector(s) play an important role in promoting certain gene expression required for cellular transformation and the upregulation of cytoskeletal changes needed for tumor cell invasion and migration. Identifica-

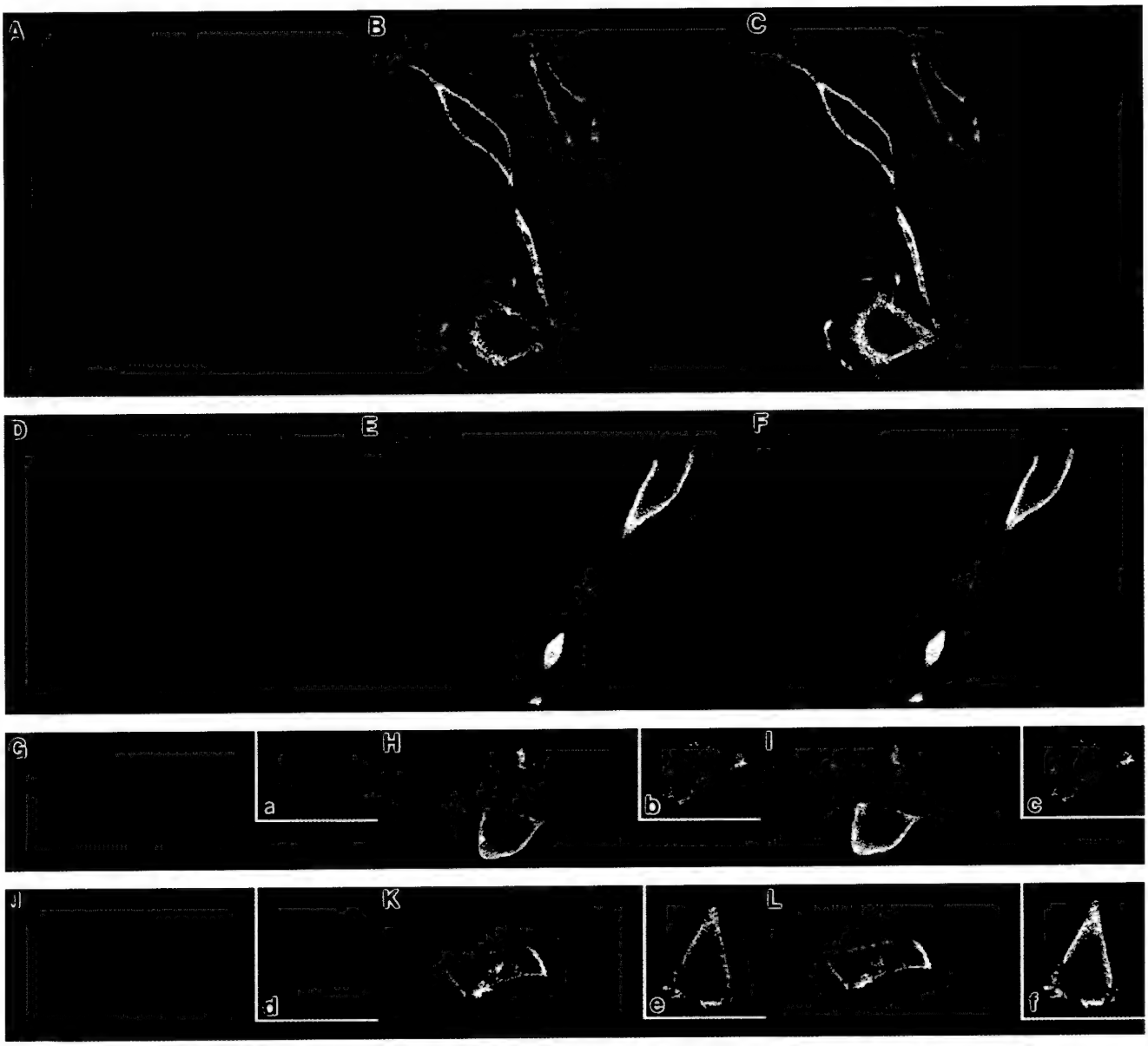

Figure 10. Double immunofluorescence staining of ankyrin and Tiam1 in untransfected SP1 cells or SP1 transfectants. SP1 cells (untransfected or transfected with HA-tagged C1199 Tiam1 cDNA or GFP-tagged Tiam1 fragment cDNA or cotransfected with HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA) were fixed by 2% paraformaldehyde. Subsequently, cells were rendered permeable by ethanol treatment and stained with various immunoreagents as described in Materials and Methods. (A–C) Rh-labeled anti-ANK3 staining (A) FITC-anti-Tiam1 staining (B) and colocalization of ankyrin and Tiam1 (C) in untransfected SP1 cells. (D–F) Rh-labeled anti-ANK3 staining (D) FITC-anti-HA–labeled C1199 Tiam1 staining (E), and colocalization of ankyrin and C1199 Tiam1 (F) in HA-tagged C1199 Tiam1 cDNA–transfected SP1 cells. (G–I) Rh-labeled anti-ANK3 staining (G), GFP-tagged Tiam1 fragment (H), and colocalization of ankyrin and Tiam1 fragment (I) in GFP-tagged Tiam1 fragment cDNA–transfected SP1 cells. (a–c) Rh-labeled normal mouse IgG staining (a), GFP-tagged Tiam1 fragment (b), and colocalization of normal mouse IgG and Tiam1 fragment (c) in GFP-tagged Tiam1 fragment cDNA–transfected SP1 cells. (J–L) Rh-labeled anti-HA staining of C1199 Tiam1 (J), GFP-tagged Tiam1 fragment (K), and colocalization of C1199 and Tiam1 fragment (L) in SP1 cells cotransfected with HA-tagged C1199 cDNA and GFP-tagged Tiam1 fragment cDNA. (d–f) Rh-labeled anti-ANK3 staining (d), GFP-tagged Tiam1 fragment (e), and colocalization of ankyrin and Tiam1 fragment (f) in SP1 cells cotransfected with HA-tagged C1199 cDNA and GFP-tagged Tiam1 fragment cDNA.

tion of immediate downstream targets for ankyrin-mediated Tiam1-Rac1 signaling is currently under investigation in our laboratory.

We have also found that SP1 cells transfected with GFP-tagged Tiam1 fragment cDNA express a 68-kD protein as detected by anti-GFP antibody (Fig. 6 B, lane 2; Fig. 9 C, b). In vector-transfected SP1 cells, we are not able to detect any protein band by anti-GFP–mediated immunoblotting (Fig. 9 A, b). Double immunofluorescence staining shows that both ankyrin (Fig. 10 G) and the GFP-tagged Tiam1 fragment (Fig. 10 H) are colocalized in the cell membranes in SP1 transfectants (Fig. 10 I). We believe that the ankyrin staining detected in these SP1 transfectants, revealed by antiankyrin-mediated immunostaining,

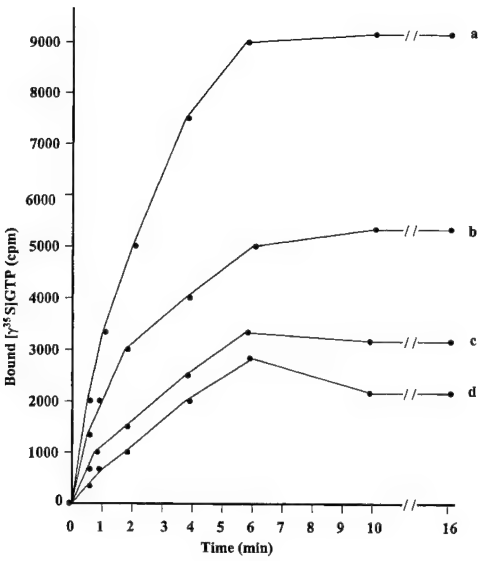

Figure 11. Kinetics of GTP-γ-^{35}S bound to GDP-loaded GST-Rac1 in the presence of ankyrin-associated Tiam1 isolated from SP-1 cells: transfected with HA-tagged C1199 Tiam1 cDNA (a) or GFP-tagged Tiam1 fragment cDNA (d); or cotransfected with HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA (c) or vector alone (b). Purified *E. coli*–derived GST-tagged GTPases (e.g., Rac1, Cdc42, or RhoA) were preloaded with GDP. First, 2 pmol ankyrin-associated Tiam1 isolated from various SP1 transfectants was added to the reaction buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 100 μM AMP-PNP, 0.5 mg/ml BSA, and 2.5 μM GTP-γ-^{35}S (~1,250, Ci/mmol). Subsequently, 2.5 pmol GDP-loaded GST-tagged Rho GTPases (e.g., Rac1, RhoA, Cdc42 or GST alone) were mixed with the reaction buffer containing ankyrin-associated Tiam1 and GTP-γ-^{35}S to initiate the exchange reaction at room temperature. At various time points, the reaction of each sample was terminated by adding ice-cold termination buffer as described in Materials and Methods. The termination reactions were filtered immediately through nitrocellulose filters, and the radioactivity associated with the filters were measured by scintillation fluid. The amount of GTP-γ-^{35}S bound to Tiam1 or control sample (preimmune serum–conjugated Sepharose beads) in the absence of Rho GTPases (e.g., Rac1, Cdc42, or RhoA) was subtracted from the original values. Data represent an average of triplicates from three to five experiments. SD < 5%.

is specific since no label (Fig. 10 a) is detected in these GFP-Tiam1 fragment–overexpressed cells (Fig. 10 b) using normal mouse IgG (Fig. 10 a). No colocalization (Fig. 10 c) of normal mouse IgG (Fig. 10 a) and GFP-Tiam1 fragment (Fig. 10 b) is observed in these transfectants. Moreover, we have demonstrated that overexpression of the GFP-tagged Tiam1 fragment in SP1 transfectants downregulates ankyrin-associated Tiam1-Rac1 signaling (Fig. 10 d), tumor cell invasion (Table II, A), as well as cell migration (Table II, B).

Finally, cotransfection of SP1 cells with HA-tagged C1199 Tiam1 cDNA and GFP-tagged Tiam1 fragment cDNA was carried out. Using anti-HA or anti GFP-mediated immunoblotting technique, we have detected coexpression of both C1199 Tiam1 (Fig. 9 D, a) and Tiam1 fragment (Fig. 9 D, b) in SP1 transfectants. In controls, no signal was detected in HA-tagged C1199 Tiam1 cDNA–transfected cells or GFP-tagged Tiam1 fragment cDNA–

Table II. Measurement of Tumor Cell Invasion and Migration

Cells	Cell invasion* DMSO-treated	Percent control‡ Cytochalasin D–treated
In vitro cell invasion		
Untransfected cells (control)	100	22
Vector-transfected cells	96	24
Tiam1 fragment cDNA–transfected cell	95	20
C1199 Tiam 1 cDNA–transfected cells	155	50
C1199 Tiam1 cDNA and Tiam1 fragment cDNA–cotransfected cells	90	17
In vitro cell migration		
Untransfected cells (control)	100	20
Vector-transfected cells	98	23
Tiam1 fragment cDNA–transfected cell	93	22
C1199 Tiam1 cDNA–transfected cells	158	55
C1199 Tiam1 cDNA and Tiam1 fragment cDNA–cotransfected cells	88	14

*SP1 cells (~10^4 cells/well in PBS, pH 7.2), in the presence or absence of 20 μg/ml cytochalasin D (dissolved in DMSO) or DMSO alone, were placed in the upper chamber of the transwell unit. In some cases, SP1 cells were transfected with either HA-tagged C1199 Tiam1 cDNA or GFP-tagged Tiam1 fragment cDNA or HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA, or vector alone. After an 18-h incubation at 37°C in a humidified 95% air/5% CO_2 atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell migration processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters containing serum by standard cell number counting assays as described in Materials and Methods. Each assay was set up in triplicate and repeated at least three times. All data were analyzed statistically by *t* test and statistical significance was set at $P < 0.01$. In these experiments ~30–40% of input cells (~10^4 cells/well) undergo in vitro cell invasion and migration in the control samples.
‡The values expressed in this table represent an average of triplicate determinations of three to five experiments with an SD less than ± 5%.

transfected cells using anti-GFP (Fig. 9 B, b) or anti-HA (Fig. 9 C, a)–mediated immunoblotting, respectively. Furthermore, immunocytochemical staining results show that ankyrin (Fig. 10 d) and the GFP-tagged Tiam1 fragment (Fig. 10 e) are colocalized (Fig. 10 f) in the plasma membranes of SP1 transfectants. In contrast, C1199 Tiam1 (Fig. 10 J) fails to display plasma membrane localization. Consequently, the level of colocalization (Fig. 10 L) between C1199 Tiam1 (Fig. 10 J) and Tiam1 fragment (Fig. 10 K) is greatly reduced. In addition, it is noted that no significant stimulation of long membrane projections was observed in these transfectants (Fig. 10, J–L and d–f). Other tumor-specific behaviors such as Tiam1-Rac1 activation (Fig. 11 c) and cytoskeleton-mediated breast tumor cell invasion (Table II, A) and migration (Table II B) are also greatly inhibited. These findings suggest that the ankyrin-binding domain-containing Tiam1 fragment acts as a dominant negative mutant that effectively competes for ankyrin binding to C1199 Tiam1 in vivo and blocks ankyrin-regulated Tiam1 function associated with tumor-specific phenotypes.

Discussion

The invasive phenotype of breast tumors, determined by

characteristics such as tumor cell motility and membrane perturbations, is clearly linked to cytoskeletal function. For example, recent studies have shown that certain metastasis-specific molecules (e.g., $CD44v_{3,8-10}$ isoform [Bourguignon et al., 1998b, 1999] and its associated matrix metalloproteinase, MMP-9 [Bourguignon et al., 1998b; Yu and Stamenkovic, 1999], as well as Rho kinase [Bourguignon et al., 1999]) are closely associated with the cytoskeleton during tumor cell function. To further examine the regulatory mechanism(s) involved in cytoskeleton-mediated oncogenic signaling leading to tumor cell invasion and migration, we have focused on GEFs (the Dbl or DH family), such as Tiam1, which are known to display oncogenic capability and function as upstream activators of Rho-like GTPases (e.g., Rac1 or Cdc42; Woods et al., 1991; Habets et al., 1994; Michiels et al., 1995; Nobes and Hall, 1995; Van Leeuwen et al., 1995). In breast tumor cells, such as SP-1 cells, Tiam1 is detected as a 200-kD protein (Fig. 1), which is similar to the Tiam1 described in other cell types (Woods and Bryant, 1991; Michiels et al., 1995; Nobes and Hall, 1995; Van Leeuwen et al., 1995, 1997; Hordijk et al., 1997; Stam et al., 1997; Bourguignon et al., 2000). Tiam1, isolated from SP-1 cells, is also capable of carrying out GDP/GTP exchange for Rac1 in vitro (Fig. 2). Sequence analysis of Tiam1 suggests that its association with the invasive and metastatic phenotype is mediated via membrane-linked cytoskeletal regulation and/or activation of Rho family GTPases (Habets et al., 1994; Nobes and Hall, 1995).

Rac1 acts downstream of Tiam1 signaling and regulates the function of several cell adhesion molecules such as the laminin receptor, $\alpha 6\beta 1$ integrin (Van Leeuwen et al., 1997), E-cadherin (Hordijk et al., 1997), and the hyaluronan receptor, CD44 (Bourguignon et al., 2000). Tiam1-Rac1 activation also has been shown to be stimulated by certain serum-derived growth activators (e.g., S1P and LPA) during T-lymphoma cell invasion (Stam et al., 1998). However, in epithelial MDCK cells, Tiam1-Rac1 signaling plays an invasion/suppressor role in Ras-transformed MDCK cells (Hordijk et al., 1997). Apparently, various responses by Tiam1-catalyzed Rac1 signaling may be controlled by selective upstream activators (e.g., availability of certain cytoskeletal proteins [e.g., ankyrin], cell adhesion receptors [e.g., CD44, integrin or E-cadherin], growth activators [e.g., serum, S1P, or LPA] or extracellular matrix components [hyaluronic acid, collagen, or fibronectin, etc.]). Moreover, Tiam1 is found to be involved in promoting both Rac1- and RhoA-mediated pathways during neurite formation in nerve cells (Van Leeuwen et al., 1997). The balance between Rac1 and RhoA determines a particular cellular morphology and migratory behavior (Sander et al., 1999).

Ankyrin is a family of membrane-associated cytoskeletal proteins expressed in a variety of biological systems including epithelial cells and tissues (Peters and Lux, 1993). Presently, at least three ankyrin genes have been identified: *ankyrin 1* (Ank 1 or *ankyrin R*), *ankyrin 2* (Ank 2 or *ankyrin B*), and *ankyrin 3* (Ank 3 or *ankyrin G*; Lux et al., 1990; Otto et al., 1991; Kordeli et al., 1995; Peters et al., 1995). These molecules belong to a family of related genes that probably arose by duplication and divergence of a common ancestral gene. Ankyrin is known to bind to a number of plasma membrane–associated proteins including the following: band 3, two other members of the anion exchange gene family (Bennet, 1992), Na^+/K^+-ATPase (Nelson and Veshnock, 1987; Zhang et al., 1998), the amiloride-sensitive Na^+ channel (Smith et al., 1991), the voltage-dependent Na^+ channel (Kordeli et al., 1995), Ca^{2+} channels (Bourguignon et al., 1993b, 1995a; Bourguignon and Jin, 1995) and the adhesion molecule CD44 (Bourguignon et al., 1986, 1991, 1992, 1993a; Kalomiris and Bourguignon, 1988, 1989; Lokeshwar and Bourguignon, 1991, 1992; Lokeshwar et al., 1994, 1996). It has been suggested that the binding of ankyrin to certain membrane-associated molecules is necessary for signal transduction, cell adhesion, membrane transport, cell growth, migration, and tumor metastasis (Bennet, 1992; Bourguignon et al., 1995b, 1996, 1997, 1998a; De Matteis and Morrow, 1998; Zhu and Bourguignon, 1998, 2000).

In this study, we have presented new evidence showing the interaction between ankyrin and Tiam1. Specifically, we have demonstrated that Tiam1 and ankyrin (e.g., ANK1 and ANK3) are physically linked to each other as a complex in vivo (Figs. 3 and 10) and in vitro (Figs. 5–7), and that ankyrin binding to Tiam1 promotes Rac1 activation (Figs. 8 and 11). Using purified Tiam1 and GST-tagged ankyrin repeat domain (GST-ARD; Fig. 5) to examine the interaction between Tiam1 and ankyrin in vitro, we have found that ARD is directly involved in the binding of Tiam1 (Fig. 5, C and D, and Fig. 6, C–E). In fact, the binding affinity of ARD to Tiam1 is very comparable to that of intact erythrocyte ankyrin binding to Tiam1 (Fig. 5, C and D). These findings support the conclusion that the ARD fragment of ankyrin is directly involved in the recognition of Tiam1. The 24 ankyrin repeats within the ARD are known to form binding sites for at least seven distinct membrane protein families (Michaely and Bennett, 1995). Often, ARD is organized into four folding subdomains: subdomain 1 (S1), subdomain 2 (S2), subdomain 3 (S3), and subdomain 4 (S4)). Recently, we have shown that the S2 subdomain, but not the other subdomains, of ARD binds to the adhesion molecule CD44 directly (Zhu and Bourguignon, 2000). Overexpression of subdomain (S2) of ARD promotes CD44-mediated tumor cell migration (Zhu and Bourguignon, 2000). The question of which ARD subdomain fragment(s) is (are) involved in regulating Tiam1 function remains to be determined.

The structural homology between the ankyrin binding domain of Tiam1 (the sequence between amino acids 717 and 727) and CD44 is quite striking (Lokeshwar et al., 1994). The cytoplasmic domain of CD44 ($\sim$70 amino acids long) is highly conserved ($\geq$90%) in most of the CD44 isoforms; and it is clearly involved in specific ankyrin binding (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998). The ankyrin-binding domain of CD44 has also been mapped using deletion mutation analyses and mammalian expression systems (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998). In particular, the ankyrin-binding domain (e.g., NGGNGTVEDRKPSEL between amino acids 306 and 320 in the mouse CD44 [Lokeshwar et al., 1994] and NSGNGAVEDRKPSGL amino acids 304 and 318 in human CD44 [Zhu and Bourguignon, 1998]) is required for cell adhesion (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998), the recruitment of Src kinase (Zhu and

Bourguignon, 1998), and the onset of tumor cell transformation (Bourguignon et al., 1998b; Zhu and Bourguignon, 1998). The facts that (1) the recombinant C1199 Tiam1 interacts with ARD fusion protein directly with a stoichiometry of 1:1 (Fig. 6, D and E); (2) a peptide with the sequence ^{717}GEGTDAVKRS^{727}L of Tiam1 binds to ankyrin and ARD but not ankyrin's spectrin binding domain or spectrin (Table I); (3) a Tiam1 peptide (amino acids 717–727) competes with Tiam1 for the binding to ankyrin (Fig. 7 A); (4) the Tiam1 deletion mutant protein (e.g., C1199 Tiam1Δ717-727; Fig. 7 B) fails to bind ankyrin (Fig. 7 D); and (5) ankyrin stimulates Tiam1-catalyzed GDP/GTP exchange activity on Rac1 (Fig. 8) strongly suggest that the sequence (^{717}GEGTDAVKRS^{727}L) of Tiam1 is an important region for ankyrin binding.

Furthermore, we have shown that transfection of SP-1 cells with HA-tagged NH_2 terminally truncated C1199 Tiam1 cDNA stimulates ankyrin-associated GDP/GTP exchange on Rac1 (Fig. 11) as well as tumor cell invasion (Table II, A) and migration (Table II, B). These Tiam1-activated oncogenic responses are consistent with previous studies indicating that Tiam1-activated Rho-like GTPases may act as downstream effectors of Ras in both tumorigenesis and progression to metastatic diseases (Habets et al., 1994, 1995; van Leeuwen et al., 1995). The amino acids 393–738 Tiam1 fragment (Fig. 6 C) contains not only the putative ankyrin-binding domain (amino acids 717–727), but also the NH_2-terminal pleckstrin homology (PHn), the coiled-coil region (CC) and an additional adjacent region (Ex) (also designated as PHn-CC-Ex domain; Michiels et al., 1997). This Tiam1 fragment has been shown to be responsible for Tiam1's membrane localization, Rac1-dependent membrane ruffling, and C-Jun NH_2-terminal kinase activation in fibroblasts and COS cells (Michiels et al., 1997; Stam et al., 1997). In this study, we have found that cotransfection of SP1 cells with Tiam1 fragment cDNA and C1199 Tiam1 cDNA effectively blocks tumor cell–specific behaviors (e.g., C1199 Tiam1 association with ankyrin in the cell membrane [Fig. 10], Rac1 activation [Fig. 11], tumor cell invasion [Table II, A], and migration [Table II B]). These findings further support our conclusion that the ankyrin-binding domain-containing Tiam1 fragment acts as a potent competitive inhibitor, which is capable of interfering with C1199 Tiam1–ankyrin interaction in vivo. Recently, we have also demonstrated that the Tiam1 fragment is required for CD44 (the hyaluronan receptor) binding (Bourguignon et al., 2000). Most importantly, Tiam1–CD44 interaction promotes Rac1 activation and hyaluronic acid–mediated breast tumor cell migration (Bourguignon et al., 2000). These observations clearly suggest that the amino acids 393–738 of Tiam1 contains multiple functional domains (e.g., membrane localization site(s) and cytoskeleton binding domains) required for the regulation of Tiam1-Rac1 signaling and cytoskeleton function. Taken together, we believe that ankyrin–Tiam1 interaction plays a pivotal role in regulating Rac1-activated oncogenic signaling and cytoskeleton-mediated metastatic breast tumor cell progression.

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

March 27, 2001

Research Data Management

SUBJECT: Review of Annual Report Dated August 2000,
Award Number DAMD17-97-1-7014

Lilly Y. Bourguignon, Ph.D.
Department of Cell Biology and Anatomy
University of Miami
1600 NW 10th Avenue
Miami, Florida 33136

Dear Doctor Bourguignon:

Subject report has been reviewed and is acceptable as written. A copy of your report has been forwarded to the Defense Technical Information Center's Technical Reports database.

To assist you in preparing future reports under the subject award, we have enclosed the reviewer's comments. We ask that you review these comments and incorporate any recommended changes into future reports.

Point of contact for this action is Ms. Judy Pawlus at 301-619-7322 or by email at judy.pawlus@det.amedd.army.mil.

Sincerely,

Judy Pawlus
Technical Editor

Enclosure

FINAL REPORT REVIEW
USAMRMC FY96 BREAST CANCER RESEARCH PROGRAM

Grant/Contract/MIPR No.:	**DAMD17-97-1-7014**
Principal Investigator:	Lilly Y.W. Bourguignon, Ph.D.
Institution:	University of Miami Miami, Florida 33101
Report Title:	A New Invasion and Metastasis Molecule, Tiam1 and Its Interaction with the Cytoskeleton Are Involved in Human Breast Cancer Progression
Report Type:	Final
Award Mechanism:	Idea
Date of Report:	August 2000
Reporting Period:	7 July 1999 - 6 July 2000

SUMMARY REVIEW: Tiam1 (T lymphoma invasion and metastasis) is a guanine nucleotide exchange factor (GEF) known by retroviral insertional mutagenesis and selected for its invasive cell behavior. Tiam1 activates Rac1 (a member of the Rho subclass of the ras superfamily) as a GEF to induce membrane cytoskeleton-mediated cell shape changes, cell adhesion, and cell motility, namely in breast tumor cells (SP-1 cell line). The overall purpose of this award is to study how this molecule is regulated in invasive and metastatic processes of breast cancer cells.

During the final year of this award, the principal investigator (PI) studied ankyrin genes known to be expressed in cytoskeletal proteins found in epithelial cells and tissues. Ankyrin binds to several proteins as well as CD44 (adhesion molecule). Ankyrin binding to certain molecules is thought to be necessary for signal transduction, cell adhesion, membrane transport, cell growth, migration, and tumor metastasis. Studies conducted by the researcher show that ankyrin binding to Tiam1 promotes Rac1 activation. However, when SP-1 cells were cotransfected with Tiam1 fragment cDNA and C1199 Tiam1 cDNA, this blocked tumor cell-specific behavior such as Rac1 activation, tumor cell invasion, and migration. It was also demonstrated that the Tiam1 fragment is required for CD44 (hyaluronan receptor) binding. The

Enclosure

Tiam1-CD44 interaction promotes Rac1 activation and hyaluronic acid-mediated breast tumor cell migration.

FORMAT/EDITORIAL ISSUES: This report conforms to USAMRMC reporting requirements.

CONTRACTUAL ISSUES: Information is provided in this final report that supports the following:

Task 1	Months 1-12	Completed
Task 2	Months 12-24	Completed
Task 3	Months 24-36	Completed

This report is in general compliance with the goals outlined in the statement of work (SOW). The PI met all goals in the SOW.

TECHNICAL ISSUES: No major technical issues were noted during the review of this report.

SPECIFIC DISCREPANCIES AND RECOMMENDATIONS: This report was very thorough and encompasses all of the work accomplished under this award. The PI prepared a very organized and easy to read report. This reviewer recommends accepting this final report.

KEY RESEARCH ACCOMPLISHMENTS: The PI has indicated unlimited distribution for the following key research accomplishments:

- Found that Tiam1 is one of the known guanine nucleotide exchange factors for RhoGTPases (Rac1) and is expressed in breast tumor cells.
- Found that ankyrin binding to Tiam1 activates GDP/GTP exchange of RhoGTPases.
- Showed that cotransfection of SP1 cells with green fluorescent protein-tagged Tiam1 fragment cDNA and Tiam1 cDNA effectively blocks Tiam1-ankyrin colocalization in the cell membrane and inhibits GDP/GTP exchange on Rac1 by ankyrin-associated Tiam1 and tumor-specific phenotypes.
- Demonstrated that transfection of SP1 cells with Tiam1 cDNA promotes Tiam1 association with CD44v3 and upregulates Rac1 signaling as well as HA/CD44v3-mediated breast tumor cell migration.
- Showed that Tiam1 contains multiple functional domains required for the regulation of Tiam1-Rac1 signaling and cytoskeleton function leading to metastatic breast tumor cell progression.

REPORTABLE OUTCOMES:

Research

Publications

- Kalish E, Iida N, Moffat FL, and Bourguignon LYW. 1999. A New CD44v3-containing isoform is involved in tumor cell migration and human breast cancer progression, *Front Biosci.* 4:1-8.
- Ameen NA, Martensson B, Bourguignon, LYW, Marino C, Isenberg J, and McLaughlin GE. 1999. CFTR channel insertion to the apical surface in rat duodenal villus epithelial cell is upregulated by cIP in vivo. *J. Cell Sci.*, 112:887-894.
- Bourguignon LYW, Zhu H, Shao L, Zhu D, and Chen YW. 1999. Rho-Kinase (ROK) promotes $CD44v_{3,8-10}$-ankyrin interaction and tumor cell migration in metastatic breast cancer cells. *Cell Motility and the Cytoskeleton*, 43:269-287.
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- Diaz F and Bourguignon LYW. 2000. Selective down-regulation of IP3 receptor subtypes by caspases and calpain during TNFα-induced apoptosis of human T-lymphoblasts. *Cell Calcium*, in press.
- Bourguignon LYW, Zhu H, Shao L, and Chen YW. Ankyrin-Tiam1 interaction promotes Rac1 signaling and metastatic breast tumor cell invasion and migration. *J. Cell Biol.*, in press.

Abstracts

- Bourguignon LYW, Zhu H, Shao L, and Chen YW. 1999. Identification of an ankyrin-binding domain in TIAM1 and its role in regulating $CD44v_{3,8-10}$-associated metastatic breast tumor cell invasion and migration. *Proceeding of the American Association for Cancer Res.*, 40:196.
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- Zhu D and Bourguignon LYW. 2000. Interaction between CD44 and the repeat domain of ankyrin promotes hyaluronic acid (HA)-mediated ovarian tumor cell migration. *Proceeding of the American Association for Cancer Res.*

- Bourguignon LYW, Zhu H, Shao L, and Chen YW. 2000. CD44 interaction with Tiam1 promotes Rac1 signaling and hyaluronic acid (HA)-mediated breast tumor cell migration. *Proceeding of the American Association for Cancer Res.*

REVISED REPORT RECOMMENDED: **YES**_____ **NO** √

REVISED SOW RECOMMENDED: **YES**_____ **NO** √